Microbial communities and pathogens in association with vegetable-processing

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To Ben, Ella and Tom,

in memoriam of my grandparents

Zusammenfassung

Kontamination von Frischeprodukten durch human- und phytopathogene Bakterien stellt eine Gefahr für die menschliche Gesundheit dar und kann zu Verlusten während der Lagerung führen. Mikrobiologische Untersuchungen von frischem Gemüse sind zu zeitintensiv, um fortlaufend während der Produktion angewendet zu werden.

In dieser Studie wurde mittels einer 16S rRNA-Gen Klonbibliothek die mikrobiologische Diversität einer karottenverarbeitenden Anlage untersucht, sowohl pathogene als auch opportunistische Gattungen wurden entdeckt. Die vierthäufigste Art gehörte zur Gattung *Arcobacter. Arcobacter* ist bekannt dafür Enteritis in Menschen zu verursachen, konnte aber bisher in Verbindung zu Frischeprodukten nicht isoliert werden. Zwei gattungsspezifische PCR-Assays wurde entwickelt: Eines spezifisch für *Arcobacter* und eines für *Pectobacterium*. Ziel war das Auftreten dieser beiden Gattungen in einer spinatverarbeitenden Anlage zu untersuchen. Die Ergebnisse deuteten daraufhin, dass beide Pathogene wiederholt auftreten. Dies wirft die Frage auf, ob pathogene und nicht-pathogene *Arcobacter*-Arten regelmäßig in der Anlage vorkommen, deshalb und um die weitere Verbreitung zu untersuchen wurde die genetische Diversität von *Arcobacter* in der gesamten Prozesslinie mittels 16S rRNA-Gen Klonbibliotheken untersucht. Die gleiche *Arcobacter*-Art wie in der karottenverarbeitenden Anlage wurde identifiziert, sowie einige unbekannte *Arcobacter*-Arten. Zusätzlich wurden auch pathogene Arten detektiert, darunter *A. butzleri* und *A. cryaerophilus*. Drei Methoden zur Detektion von Pathogenen wurden entwickelt und bewertet:

1. Eine Multiplex-PCR wurde konstruiert, die es ermöglicht neun *Arcobacter*-Arten simultan zu unterscheiden.

2. Zwei qPCR-Assays wurden entwickelt und getestet, die eine Quantifizierung der Bakterien ermöglichen.

3. MALDI-TOF MS ermöglicht eine schnelle und präzise Identifizierung von Bakterien. Das Potential dieser Methode wurde getestet durch die Bestimmung der mikrobiellen Diversität der spinatverarbeitenden Anlage und durch die spezifische Detektion von *Arcobacter*.

Abstract

Contamination of produce by human- and phytopathogenic microorganisms may result in high losses during storage and poses a threat for the human health. Microbiological examination of the extent of contamination is time-consuming and therefore cannot be applied routinely during processing of fresh vegetables.

In this study, the microbial diversity of a carrot-processing plant was evaluated by construction of a 16S rRNA gene clone library. Several species of pathogenic or opportunistic genera were detected. The fourth most common species belonged to *Arcobacter*. *Arcobacter* is known for causing enteritis in humans, but has not, until recently, been associated with the production of vegetables.

Two genus-specific PCR-Assays were developed: One specific for *Arcobacter* and one for *Pectobacterium*. The aim was to ascertain the occurrence of *Arcobacter* and *Pectobacterium* in a spinach-processing plant. Results indicated a repeated occurrence of both pathogens in the spinach-processing line, which raised the question whether pathogenic and non-pathogenic *Arcobacter* spp. are a common occurrence in vegetable-processing plants. To get further information about the dispersion of the bacteria, the genetic diversity of *Arcobacter* in the entire process line was established by 16S rRNA gene clone libraries. The same *Arcobacter* species as in the carrot-processing plant were detected, as well as other unknown *Arcobacter* species. Additionally, several pathogenic species were identified, including *A. butzleri* and *A. cryaerophilus*.

Three different methods for detection of pathogens were developed and evaluated:

1. A multiplex-PCR was designed making it possible to detect nine *Arcobacter* species simultaneously.

2. Two qPCR-Assay specific for *Arcobacter* and *Pectobacterium* were developed and tested to enable enumeration of the bacteria.

3. MALDI-TOF MS is a culture-dependent method to identify and enumerate species fast and accurate. The potential of this method was evaluated by establishing the microbial diversity of the spinach-processing line and by specific detection of *Arcobacter*.

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Abbreviations

ARDRA	amplified rDNA restriction analysis
cfu	colony-forming unit
CTAB	cetyltrimethylammonium bromide
DAPI	4',6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
EHEC	enterohemorrhagic E. coli
HUS	hemolytic-uremic syndrome
IPTG	Isopropylthiogalactopyranoside
MALDI-TOF MS	matrix-assisted laser desorption/ ionisation - time-of-flight mass spectrometry
NTC	no-template control
PCR	Polymerase chain reaction
PCR	quantitative PCR
PVPP	Polyvinylpolypyrrolidone
rDNA	ribosomal RNA gene
SLS	samples loading solution
TE-Puffer	Tris-EDTA-Puffer
TRIS	Tris(hydroxymethyl)aminomethane
VBNC	viable-but-not-culturable
x-Gal	bromo-chloro-indoyl-galactopyranoside

1. Introduction

Food-borne diseases caused by bacteria and viruses continue to present a serious health threat. Once again this became apparent in Germany where one of the largest outbreaks worldwide of an enterohemorrhagic *Escherichia coli* (EHEC) occurred. More than 4,300 people were infected, 859 of whom actually developed or were suspected of developing hemolytic-uremic syndrome (HUS). The accounts of deaths attributed to HUS amounted to 48. In other countries of the EU additional 124 EHEC infections were reported, 48 of whom developed HUS and one of whom died (EFSA, 2011a).

Shortly after it became clear that an epidemic took place, the focus of all investigations for the source of the infections was on vegetables as the transmitting vector. An extensive search was conducted and after several weeks fenugreek sprouts were identified as the source of the outbreak (EFSA, 2011b).

The causing EHEC strain (LB226692) was sequenced shortly after the outbreak started (Mellmann et al., 2011). It has the serotype O104:H4 and is closely related to strain HUSEC041, which has been isolated before in Germany in 2001 (Mellmann et al., 2011), in France in 2004, in South Korea in 2005 (Bae et al., 2006), in Georgia in 2009 and in Finland in 2010 (Struelens et al., 2011). Strain LB226692 causing the recent outbreak is also related to EAEC O104:H4 strain 55989 (Mellmann et al., 2011). Until now, EHEC O104:H4 had seldom been isolated from HUS patients.

In the past, nearly 50 % of all HUS cases in Germany and 95 % of all cases in North America were caused by strain O157:H7 (Karch et al., 2005). The sudden emergence of O104:H4 as a serious pathogen and origin of an epidemic shows that it is nearly impossible to predict the occurrence of epidemics, their origins and causes.

An elaborate study of the European Food Safety Authority (EFSA) traced the fenugreek seeds of the contaminated sprouts back to Egypt where they had been produced. One charge of 15,000 kg was exported in 2009 to a German importer who sold a part of the seeds to a German farm which produced the sprouts. This was the source for the German cases of EHEC infection and many of the cases in other countries which have directly been connected to the German outbreak (EFSA, 2011b). At the same time another outbreak occurred in France. The German importer had sold another portion of the seeds to a British distributor who repacked them and

sold them to France.

This shows how easily a disease can spread across country borders along transportation and trade routes. Although health agencies were finally able to identify the source of the outbreak and were able to reconstruct the transport routes, in only one package of sprouts an EHEC contamination was detected. It was argued that the EHEC cells had entered a viable-but-not-culturable (VBNC) state and are not growing in the enrichment media typically used for this purpose, but do grow significantly in the human digestive system (EFSA, 2011b).

With its source, its origin, its distribution and difficult detection, the last EHEC epidemic is an example of the kind of food-borne illnesses which have been observed in increasing numbers during the past years (Olsen et al., 2000) and represent an enormous challenge for the health agencies and the food industry. In 2007 1,098 food-borne outbreaks were reported in the U.S provoking in total 21,250 illnesses, 904 hospitalizations, and 18 deaths (CDC-Outbreak-Database). For 67 % of these outbreaks no etiology was identified and in 53 % of them the specific vehicle was not found. (See Table 1.1 for the number of outbreaks with known etiology and food commodity.) Mead et al. (2000) estimated that every year food-borne pathogens cause 76 million illnesses in the U. S., leading to 325,000 hospitalizations, and 5,000 deaths. This estimation was challenged by Hedberg (1999), but even if the actual numbers are lower, the impact on the national health systems and economies is considerable. In the U. S. 12 % of food-borne illnesses reported from 1990 to 2003 were linked to produce (DeWaal et al., 2006).

However, not only absolute numbers of incidents are significant; in recent years an increase of food-borne illness incidents has been reported (Beuchat & Ryu, 1997). This may be attributed to heightened awareness and to better detection methods, but also to changes in lifestyle in the last years. The health awareness of the customer has risen and more fresh food is consumed (Hedberg et al., 1994). Additionally, the transportation routes became longer to meet the requirements of a global market, which satisfies the need for fresh vegetables and fruits throughout the year (Beuchat, 2002).

Vegetables and fruits are not only contaminated with human pathogens, but also with phytopathogens, which results in an estimated 24 % of post harvest losses in the U.S. (Agric., 1965) and 50 % of losses of harvested crops worldwide (Wilson & Wisniewski, 1989).

A routine control of vegetables for human pathogens and spoilage bacteria is not feasible as reference methods of the regulatory agencies are based on microbiological methods and typically are too time-consuming for fresh food, which has to be processed and consumed in a matter of days. Additionally, all plating methods are inherently biased because bacteria can survive in a viable-but-not-culturable state (Oliver, 2010). New methods have to be developed that enable fast and reliable detection. Unfortunately, little is known about the microbiology of vegetable-processing so that a reliable threat assessment is impossible.

Table 1.1.: Number of reported food borne disease outbreaks and outbreak-associated illnesses, by etiology and food commodity – United States, 2007. Table modified from CDC-Outbreak Surveillance Data (CDC-Outbreak-Database)

	Beans-Grains	Oils-Sugars	Fruits-Nuts	Leafy Vegetables	Root	Sprout	Vine-Stalk
Bacterial							
Salmonella sp. §	1 (3)	1 (14)	1 (11)	2 (87)	- -	3 (59)	2 (88)
Clostridium perfringens	2 (130)	- -	- -	- -	- -	- -	- -
Staphylococcus sp., enterotoxin ¶	1 (9)	- -	- -	- -	- -	- -	- -
Escherichia coli, Shiga toxin-producing**	- -	- -	1 (9)	2 (34)	- -	- -	- -
Campylobacter sp. ††	- -	1 (3)	- -	1 (26)	- -	- -	- -
Bacillus cereus	2 (8)	- -	- -	- -	- -	- -	- -
Shigella sp. §§	- -	- -	- -	2 (116)	- -	- -	- -
Clostridium botulinum	- -	- -	- -	- -	- -	- -	1 (4)
Bacterial total	6 (150)	2 (17)	2 (20)	7 (263)	- -	3 (59)	3 (92)
Viral							
Norovirus	- -	1 (5)	7 (197)	13 (315)	2 (30)		2 (35)
Hepatitis A	- -	- -	1 (3)	- -	- -	- -	- -
Viral Total		1 (5)	8 (200)	13 (315)	2 (30)		2 (35)
Single Etiology (subtotal)	6 (150)	3 (22)	10 (220)	20 (578)	2 (30)	3 (59)	5 (127)
Unknown Etiology***	4 (135)	- -	5 (35)	1 (4)	1 (5)	- -	1 (28)
Multiple Etiologies	2 (15)	- -	- -	1 (8)	- -	- -	- -
Total 2007	12 (300)	3 (22)	15 (255)	22 (590)	3 (35)	3 (59)	6 (155)

§ *Salmonella* serotypes accounting for more than five outbreaks reported include: Enteriditis (30 outbreaks), Typhimurium (20), Newport (17), and Heidelberg (9), and Montevideo (9).

¶ *Staphylococcus aureus* (11 confirmed outbreaks, 9 suspected outbreaks) and *Staphylococcus* unknown (1 suspected outbreak)

** STEC O111 (1 confirmed outbreak), STEC O157:H7 (36 confirmed outbreaks, 2 suspected outbreaks), and STEC O157:NM(H-) (3 confirmed outbreaks)

†† *Campylobacter jejuni* (14 confirmed outbreaks, 3 suspected outbreaks) and *Campylobacter* unknown (7 confirmed outbreaks, 3 suspected outbreaks)

§§ *Shigella sonnei* (9 confirmed outbreaks, 1 suspected outbreak) and *Shigella* unknown (1 confirmed outbreak)

¶¶ *Listeria monocytogenes* (1 confirmed outbreak)

*** An etiologic agent was not confirmed or suspected based on clinical, laboratory or epidemiologic information.

In this study, the microbial diversity of a carrot-processing plant was evaluated by construction of a 16S rRNA gene clone library (see Section 4.1). After the most common bacteria were identified two genus-specific polymerase chain reaction (PCR) assays were developed to facilitate detection: One specific for *Arcobacter* as an example of a human pathogen and one for *Pectobacterium* as a spoilage bacterium (see Section 4.2). The PCR protocols were tested on samples of a spinach-washing plant with a more complex processing line than the carrot-washing plant. The genetic diversity of *Arcobacter* in the entire processing line was established by 16S rRNA gene clone libraries. Analysis of the clone libraries provided information about the occurrence and the dispersion pattern of pathogenic and non-pathogenic *Arcobacter* species (see Section 4.3).

On the basis of the diversity analysis three different detection methods were developed and tested:

1. A multiplex-PCR was developed to enable the detection of several *Arcobacter* species simultaneously and tested on samples from the spinach-processing line (see Section 4.4). As the visualization of many different fragments with gel electrophoresis proved to be difficult, because of problems with resolution, capillary electrophoresis was tested as a new visualization method.

2. Quantitative PCR (qPCR) assays for the detection of *Arcobacter* and *Pectobacterium* were developed to enable quantification (see Section 4.2 and 4.6). Even though, qPCR is a sensitive and accurate method, it unfortunately does not have much potential for multiplex assays. Therefore, the qPCR-Assays were designed to be genus-specific.

3. In recent years matrix-assisted laser desorption/ ionization-time-of-flight mass spectrometry (MALDI-TOF MS) has been optimized for the detection and quantification of bacteria with good results. After plating of samples, colonies are picked and measured in the MALDI-TOF analyzer. The resulting spectra of all proteins are used for identification. In this study, this method was tested for its potential in establishing the microbial diversity and for the specific detection of *Arcobacter* (see Section 4.7).

2. Literature review

Fruits and vegetables can be exposed to pathogenic microorganisms in a multitude of ways during growth, processing and distribution. Before harvest one source of contamination with bacteria, viruses and parasites may be the soil where the produce is grown in which may be contaminated by animals, insects, dust, irrigation water (Franz & van Bruggen, 2008) and the application of not sufficiently composted sewage or manure (Franz & van Bruggen, 2008, De Roever, 1998). In particular, irrigation water is a major potential source of contamination. In Germany $85\,\% - 95\,\%$ of the water used by farmers on their fields is taken from surface water like rivers and lakes (Fröhlingsdorf et al., 2011). Most waste water treatment plants are not sufficiently equipped to remove pathogens from sewage water (Crockett, 2007). In case of intense rain, water is released from the canalization without treatment into the rivers to prevent flooding in the cities, which means that pathogens in the fecal matter, although diluted by the rain, might find their way into the surface waters used for irrigation (Fröhlingsdorf et al., 2011).

Post harvest contamination may occur by human handling, use of contaminated equipment, transport containers, animals, dust, wash water, ice, improper storage and packaging (Beuchat, 1996). The evaluation of potential routes of transmission and sources of contamination is difficult since – while there are common factors in the production of fresh produce – there are also many differences between the products like their physical characteristics, growing and harvesting practices, cooling techniques, and optimal storage temperature and environment (De Roever, 1998). As every vegetable has its own production process it is difficult to develop control points and methods which can be applied to all or at least to several of them. For example, while most vegetables and fruits are washed, this does not apply to berry fruits which do not react well to the contact with water (De Roever, 1998).

In particular, the production of sprouts which have been involved in many outbreaks in the past and which were the cause of the last outbreak of EHEC in Germany in 2011 presents some hygienic issues: For germination sprouts are kept moist and at warm temperatures, which are ideal for the growth of bacteria. Most decontamination procedures would effect the germination capability of the seeds (on Microbiological Criteria for Foods, 1999) and can therefore not be applied. In most outbreaks in recent years the origins were sprouts, leafy vegetables and unpasteurized apple juice. Generally, nearly every kind of vegetable has been associated with an

outbreak in the past (De Roever, 1998, Beuchat, 1996).

Nguyen-the & Carlin (1994) were able to show that the microflora on produce on the market greatly reflects the bacteria present at the time of harvest. In many cases contamination with food-borne diseases occurred during growing and harvesting (De Roever, 1998). Decontamination after harvest and before processing or selling the produce should therefore decrease the risk greatly. Produce is often washed with chlorinated water to remove dirt and bacteria, but it has been shown that this treatment reduces the bacterial load only by $90\,\% - 99\,\%$. Similar numbers are obtained in laboratory studies for other sanitizing agents (Sapers, 2001). In many cases vegetables are only washed in tap water. Another important factor is the time between contamination and washing (Sapers, 2001). It was shown by Sapers (2001) that 24 h after contamination *Escherichia coli* cells were attached to the surface of apples so strongly, that they could not be removed by washing. In contrast, 30 minutes after contamination it was possible to reduce their concentration by $90\,\%$.

Many bacteria grow in biofilms covering the equipment. A biofilm is an extracellular polysaccharide matrix that keeps the bacteria attached to each other and the surface. Thus they are protected against sanitizing agents and detachment. Several human- and phytopathogens are known to have the ability of forming biofilms, e.g. *E. coli* O157:H7, *Salmonella*, *Listeria monocytogenes* and *Pseudomonas* (Brooks & Flint, 2008).

The washing equipment differ greatly (Sapers, 2001), but many re-use the water to reduce costs and prevent wasting of tap water. This increases the risk of spreading a pathogen from one batch of vegetables to the next (Nguyen-the & Carlin, 1994). It was also shown that fruits and vegetables are able to internalize pathogens, especially if they are washed with contaminated water (Sapers, 2001, Hudson & Turner, 2002).

2.1. Human pathogens on produce

According to EFSA in 2009 4.4 % of food-borne illnesses were reported which were associated with fruit, vegetables, their juices, and their products. The most common causative agents were viruses and the bacterium *Clostridium* (see Figure 2.1). Compared to all other foods, vegetables and fruits play a minor role as vehicles in food-borne illnesses in Europe. But it seems like this may change as can be seen by the recent outbreak of EHEC in Germany. In the years 1998 to 2007 produce was the second most common origin of food associated outbreaks after seafood in the US at a rate of 14.7 % (DeWaal et al., 2009). In 2007, 13 % of all outbreaks with 1173 illnesses were transmitted by produce or related products (CSPI Outbreak database).

In general, the most common pathogens which have been identified as the source of food-borne illnesses are: *Escherichia coli, Campylobacter spp., Yersinia enterocolitica, Listeria monocytogenes, Staphylococcus aureus*. Additionally, vegetables are often contaminated with

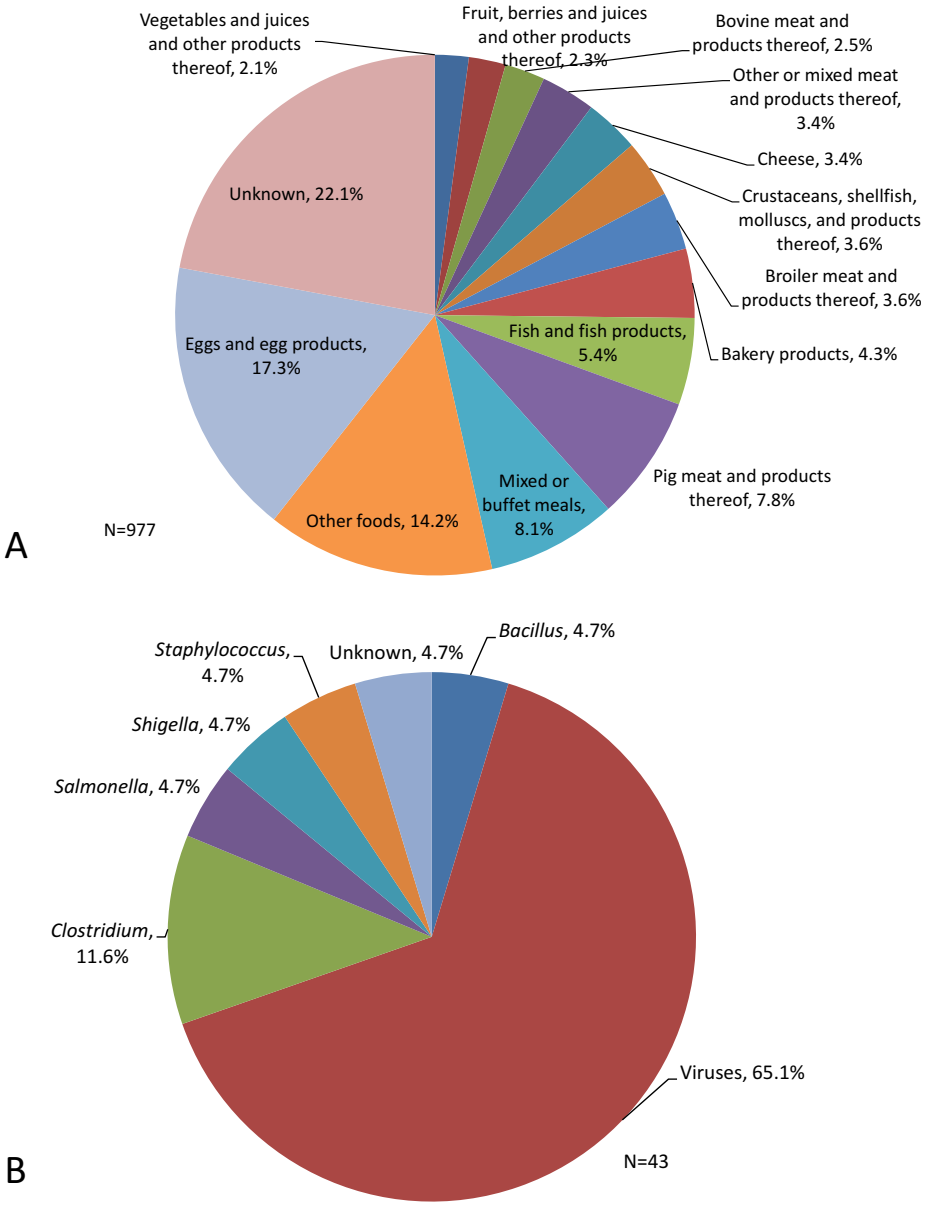

Figure 2.1.: Causative vector and etiologies of food-borne outbreaks in the EU in 2009. (A) Distribution of verified outbreaks by food vehicle in EU, 2009. (B) Distribution of causative agents in verified outbreaks caused by fruit and vegetables in EU, 2009 (Anonymous, 2011). Other foodstuffs (N=139) include: other or unspecified poultry meat and products thereof (17), dairy products (other than cheeses) (13), cereal products including rice and seeds/pulses (nuts, almonds) (11), turkey meat and products thereof (5), milk (4), herbs and spices (2), sheep meat and products thereof (2), sweets and chocolate (2) and other foods (83).

spores of *Bacillus cereus, Clostridium botulinum* or *Clostridium perfringens*. Besides the bacterial pathogens there are also viruses like Hepatitis A and Norwalk viruses and parasites like *Gardia lamblia* which are associated with outbreaks (Beuchat, 1996).

2.2. Phytopathogens on produce

Post harvest losses of agricultural products amount to large fractions of the entire harvest. Depending on the type of food, the climate, and methods of processing post harvest losses may vary significantly in different parts of the world, and it is very difficult to estimate the exact amount of losses (Wilson & Wisniewski, 1989). The Food and Agriculture Organization of the United Nations (FAO) estimates that $15\,\% - 50\,\%$ are lost, but it is not clear how much may be contributed to wastefulness, to losses during production, to animals or to spoilage. Reducing post harvest losses would contribute to the efforts that are made increasing the world food supply and to reduce hunger (Food and Agriculture organization).

Spoilage of food by microorganisms is mostly contributed to pectolytic enzyme producing bacteria, lactic bacteria, molds, and yeasts. They are part of the microbial flora of produce. Soft rod is the most important cause of spoilage of produce and is typically linked to *Pectobacterium* and *Pseudomonas*. To date, these are the only bacteria known to have the ability to produce pectate lyases which they use to degrade the middle lamella and cell walls of the plant cells (Toth et al., 2003, Perombelon, 2002). *Xanthomonas*, *Clostridium*, *Bacillus* and *Cytophaga* follow on the settlement of pectolytic bacteria as secondary infections.

Besides the direct losses caused by spoilage bacteria, microorganisms may also pose a threat in combination with human pathogens. The observed influence of co-contamination of produce with human- and phytopathogens varies. Wells & Butterfield (1997, 1999) showed that twice as much *Salmonella* were isolated from spoiled produce than from healthy produce and that the occurrence of *Salmonella* increased 5 to 10 fold on potato and carrot slices when co-inoculated with *Pectobacterium* or *Pseudomonas*. These results were supported by Carlin *et al.* (Carlin et al., 1995) who observed similar effects on *Listera monocytogenes*. In contrast, Babic et al. (1996) reported an antagonistic effect of contamination with phytopathogens on *Listera monocytogenes*.

2.3. Detection of bacteria by culture-dependent methods

Classical culture-dependent methods are suitable for the detection and enumeration of bacteria. They are based on the principle to count the colonies grown on nutrient media. These culture-based methods are accepted as reference methods suited for official controls. They are widely used by official control agencies and are internationally recognized (Jasson et al., 2010). Culture-

based methods are used as reference methods and represent the "gold-standard" of microbial analysis and are defined in the Commission Regulation (EC) No. 1441/2007 amending Regulation No. 2017/2005 on microbiological criteria for foodstuffs (European Commission, 2007) (see Table 2.1).

Advantages of culture-based methods are that the protocols are freely available and the required consumables are cheap. The methods are internationally accepted and an important foundation for international trade (Jasson et al., 2010).

2.3.1. Plate-count methods

For plate-count methods a sample is plated on selective media, so that cells can multiply and form colonies, which can be counted with the naked-eye. Before a sample can be spread on a plate other methods are often needed, for example enrichment of the targeted organism.

After enrichment, a portion of the sample is spread on a plate (spread-plate method) or mixed with the media and poured onto a plate (pour-plate method). After an incubation period of at least one night at temperatures of 7 °C – 55 °C and atmospheric conditions (aerobic, anaerobic or microaerobic) specific to the organism the grown colonies are counted. Each colony represents a colony-forming unit (cfu) usually derived from one cell. After successful isolation of a microorganism, it has to be confirmed and classified phenomenologically.

Colony-count methods have a quantification limit of $4\,\mathrm{cfu\,ml^{-1}}$ in liquid foods or $40\,\mathrm{cfu\,g^{-1}}$ in solid foods.

On the other hand they use a lot of – however not expensive – resources in form of media, liquids and processing time. For the food industry, especially regarding fresh produce, the methods often take too long to identify contaminations (Rosmini et al., 2004).

Culture-based methods can be used to identify certain pathogens or bacteria with selective-media and also to establish the microbial diversity of habitats with certain limitations. Their greatest disadvantage for accurate ecologic research is their strong bias. It has been estimated that only 1 % of all bacteria are cultivable (Amann et al., 1995, Amann & Ludwig, 2000). There is an ongoing discussion about the validity of this estimate. Recent studies confirm that microbial and molecular methods detect only a portion of the diversity and should be used in combination in order to obtain an exhaustive picture of microbial diversity (Donachie et al., 2007). Additionally, culture-dependent methods are biased by the viable-but-not-culturable (VBNC) state of some bacteria (Oliver, 2010).

Table 2.1.: Food safety criteria, from the The Commission of the European Communities

Food category	Microorganisms/ their toxins, metabolites	Sampling plan (1)		Limits (2)		Analytical reference method	Stage where the criterion applies
		n	*c*	*m*	*M*		
Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes	*Listeria monocytogenes*	10	0	Absence in 25 g		ISO 11290-1	Products placed on the market during their shelf-life
Ready-to-eat foods able to support the growth of L. monocytogenes, other than those intended for infants and for special medical purposes	*Listeria monocytogenes*	5	0	100 cfu/g		EN/ISO 11290-2	
Sprouted seeds (ready-to-eat)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	
Precut fruit and vegetables (ready-to-eat)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	
Unpasteurized fruit and vegetable juices (ready-to eat)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	
Precut fruit and vegetables (ready-to-eat)	*E. coli*	5	2	100 $\text{cfu} * \text{g}^{-1}$	1000 $\text{cfu} * \text{g}^{-1}$	ISO 16649-1 or 2	Manufacturing process
Unpasteurized fruit and vegetable juices (ready-to-eat)	*E. coli*	5	2	100 $\text{cfu} * \text{g}^{-1}$	1000 $\text{cfu} * \text{g}^{-1}$	ISO 16649-1 or 2	

(1) *n* number of units comprising the sample; *c* number of sample units giving values between *m* and *M*.
(2) *E. coli* in per-cut fruit and vegetables (ready-to-eat) and in unpasteurised fruit and vegetable juices (ready-to-eat):
— satisfactory, if all the values observed are < m,
— acceptable, if a maximum of c/n values are between m and M, and the rest of the values observed are $\leq$ m,
— unsatisfactory, if one or more of the values observed are >M or more than c/n values are between m and M.

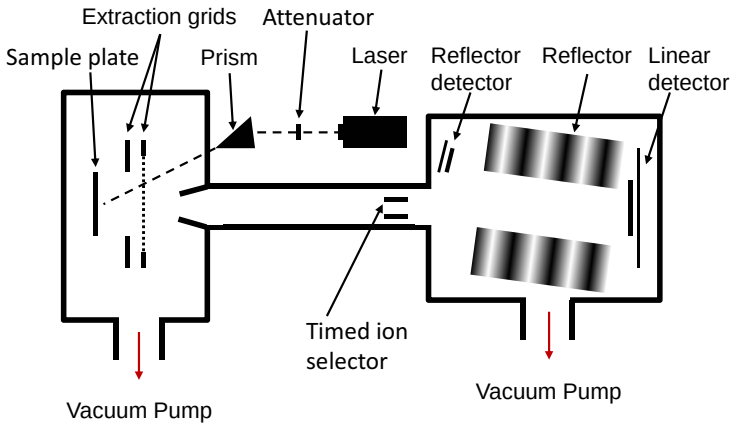

Figure 2.2.: Schematic of a MALDI-TOF analyzer with a linear and reflectron MALDI-TOF. Bacterial colonies are placed on the sample plate. The laser beam fragmentizes the cells and ionizes the fragments, which start to move, in linear mode, through the extraction grids and the field free tube directly to the detector. The extraction grids are used for the Delayed Extraction. In reflectron mode the ions are reflected and move to the reflector detector. Pumps maintain a vacuum in the analyzer.

2.3.2. Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS)

Since the 1970's MALDI-TOF MS has been used for many different applications like identification of organic and anorganic pollutants in air, water and biologic samples. In 1994, Cain *et al.* (1994) described a method to identify bacteria by MALDI-TOF MS. Proteins isolated from crude extracts were measured by MALDI-TOF MS and it was possible to distinguish several bacterial species. This new field of application was rapidly developed and identification of bacteria as intact cells became possible (Holland et al., 1996, Claydon et al., 1996).

Principle of MALDI-TOF MS The method is based on the ionization of the proteins in a cell by a laser pulse and subsequent acceleration of the ions. Depending on the mass-to-charge ratio ions reach a detector which measures the time of flight. This results in a spectra representing the various proteins in a cell. 70 % of the mass-to-charge ratio (m/z) of a spectrum represent ribosomal proteins. The spectra can be compared to reference spectra or a superspectrum. A superspectrum is generated from all spectra of a species (spectra of about 15 . . . 20 strains) and represents the major peaks defining the species.

The sample is placed on a plate and covered with a matrix-solution like 2,5-dihydroxy benzoic acid (DHB) or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SIA). The matrix crystallizes and the analyte is incorporated into the crystal structure. This prevents the destruction of the

sample molecules during ionization with the laser (Karas & Hillenkamp, 1988). During ionization protons are transferred from the matrix to the sample molecules. Ions are accelerated by an electric field before they move through an field-free channel. Here they separate according to their mass-to-charge ratio and impact on the detector after a specific time of flight (see Figure 2.2).

Ions with similar masses can have different velocities because energy is unevenly distributed during ionization. This effect can be compensated by Delayed Extraction (DE), where the ions are first allowed to move according to their kinetic energy. Ions with more energy move faster from the source. After a short delay the extraction pulse is applied and transmits more energy to the ions. The ions that had less energy and moved slower are still nearer to the source and receive more energy than the faster ions. The result is that the energy differences are compensated and ions with the same mass reach the detector at the same time (de Hoffmann & Stroobant, 2007).

Another method to compensate for the initial energy spread of ions with similar masses is a reflectron. A reflectron is an ion mirror, that reflects the ions to a second detector (reflector detector). Ions with a higher energy move farther into the reflector area before they are reflected as ions with less energy. Thus the faster ions travel a longer way to the deflector than the slower ions. This increases the resolution as well. Modern MALDI-TOF mass spectrometer combine a linear MALDI-TOF with DE and a reflectron MALDI-TOF in a single device (Mamyrin, 1994).

Detection of microorganisms with MALDI-TOF Recently, several studies explored whether MALDI-TOF MS is an alternative method for taxonomical and economical applications in contrast to or alongside genomic and classic identification methods. Several studies showed that MALDI-TOF is a reliable, accurate and reproducible method. In comparison with 16S rRNA gene sequencing, MALDI-TOF MS showed similar results, in some cases it was even superior and enabled a taxonomic classification to the subspecies level (e.g. Vorob'eva et al., 2011).

MALDI-TOF MS has successfully been used for the identification of bacteria like *Staphylococcus aureus* and it was possible to differentiate between methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* strains (Walker et al., 2002, Edwards-Jones et al., 2000). In case of *Streptococcus* a discrimination of the Lancefield groups of the ß-hemolytic streptococci was possible (Lartigue et al., 2009), while Friedrichs *et al.* (2007) were able to correctly identify 99 isolates.

Several other studies tested MALDI-TOF MS for its ability to accurately classify bacteria and other organisms, e.g.

- *Salmonella* sp. (Leuschner et al., 2004, Dieckmann et al., 2008),
- *Listeria* sp. (Barbuddhe et al., 2008),
- *Lactococcus* sp. (Tanigawa et al., 2010),

- *Neisseria* sp. (Ilina et al., 2009),
- *Aeromonas* sp. (Donohue et al., 2006),
- *Campylobacter* sp. (Fagerquist et al., 2006),
- *Helicobacter* sp. (Ilina et al., 2010),
- *Fungi* sp. (Ferreira et al., 2011b), and
- *Nematodes* sp. (Perera et al., 2005).

Several studies report that it was possible to identify bacteria directly in blood and urine cultures with MALDI-TOF MS (Ferreira et al., 2011a, Kroumova et al., 2011). Christner et al. (2010) were even able to positively identified two isolates when they were mixed in the blood culture. This is not an exhaustive list of studies using MALDI-TOF MS as a detection method of microorganisms as it has become a very active field of research and new studies are regularly published.

Study of microbial diversity with MALDI-TOF Ecologic studies with MALDI-TOF MS are rare. Most studies concentrate on the applicability of the method for the detection of bacteria, especially pathogens and its discriminative power regarding taxonomic classification. So far, only Munoz *et al*. (2011) used it to analyze isolates from hypersaline sediments of a solar saltern. The isolates were separately obtained under six different culture conditions to reduce the bias introduced by the use of only one medium.

One major disadvantage of MALDI-TOF MS is that it depends on cultivation of microorganisms and is subject to the same bias as all culture-based methods, namely that cells that are not cultivable cannot be isolated and analyzed.

2.4. Culture-independent methods

The disadvantages of culture-dependent methods can be compensated by molecular methods which are rapid and sensitive. As time for growth and identification is not required, methods like PCR are faster (Girones et al., 2010). In some cases PCR is accepted as a reference method for regulatory agencies (Malorny et al., 2003), e.g. for the detection of *Salmonella*.

2.4.1. Polymerase chain reaction

Polymerase chain reaction (PCR) was developed by Kary Mullis (Mullis & Faloona, 1987). It uses the DNA polymerase to amplify a region of target-DNA that is defined by a given set of

primers *in vitro*. Primers are short oligonucleotides with a typical length of 20 bp−30 bp that have a complementary sequence to the ends of the target region. A PCR cycle consists of a step heating the DNA solution to over 94 °C in order to separate the DNA double strands. Then the temperature is reduced to about 50 °C – 60 °C in order to allow annealing of the primer pair. The sequence of the primer pair defines the annealing temperature. In a third step a temperature is chosen (72 °C – 78 °C) at which the polymerase shows its optimal activity. At this step, called elongation, the polymerase assembles the fragment between the two primers.

The steps from strand-separation to elongation are repeated 20 to 40 times which allows for an exponential amplification of the target gene. It was shown that the detection limit can be as low as one to five cells in a sample of 100 ml (Bej et al., 1990).

PCR has been extensively used in the course of the last years for a multitude of applications. In particular, it is useful for the detection of pathogens, since PCR is very specific and sensitive. On the other hand PCR verifies the presence of DNA of both living and dead cells and does not allow for an assessment of cell viability and might lead to false positive results (Wang & Levin, 2006). It is known that DNA is very stable even after destruction of the cell depending on the environment (Josephson et al., 1993, Masters et al., 1994). Additionally, results obtained by PCR may be distorted if the PCR is inhibited by substances in the sample like humic acids, organic matter and clay particles which may have been isolated together with the nucleic acids during DNA isolation (Kirk et al., 2004).

PCR has also proven very useful for the determination of microbial diversity of different habitats. For this application the sequence analysis of the bacterial 16S rRNA gene is widely applied (Woese, 1987, Lane et al., 1985). The 16S rRNA gene is highly conserved and is ubiquitous in Bacteria and Archaea. The conserved regions allow for the design of primers specific for many taxonomic groups, while the variable regions provide the means to compare and distinguish different species (Woese, 1987). Unfortunately, the 16S rRNA gene is, in some cases, too conserved to allow for a reliable separation of species in distinct taxonomic groups such as Enterobacteriaceae (Case et al., 2007).

Ecologic studies with PCR may have major pitfalls: special care has to be taken to ensure that samples are stored at low temperatures and that DNA is extracted as quickly as possible, otherwise the microbial composition may change (von Wintzingerode et al., 1997). PCR also depends on the extraction of a sufficient amount of nucleic acids. A strong bias may be introduced, if not all cells are destroyed during lysis. Bacteria which readily undergo lysis would preferably be isolated, while too harsh conditions would lead to a destruction of DNA from readily lysed cells (von Wintzingerode et al., 1997). Primers themselves may introduce a bias as they may favor certain taxonomic groups due to their specific sequence (Sipos et al., 2007).

2.4.2. Multiplex-PCR

Multiple microorganisms can be detected with a multiplex-PCR (mPCR) which can amplify several target genes in one PCR run (Kawasaki et al., 2009). The greatest challenge in assay design is the possibility that primer dimers may be formed which potentially reduces sensitivity (Gilbride et al., 2006). Additionally, the risk of cross-amplification of primers may be increased with each primer added to the PCR mix which may lead to false positive results. For example, a primer pair was designed for bacterium A and another for bacterium B. This could lead to the situation that forward primer A might produce an amplicon with reverse primer B from the DNA of bacterium C.

Capillary gel electrophoresis The applicability of multiplex-PCRs can be increased, if capillary electrophoresis (CE) is used to analyze the amplified PCR products. CE has a high separation efficiency and is useful for high-throughput analyses (Kleparnik & Bocek, 2007). It is able to separate fragments which differ in length by only 1 bp. Capillary electrophoresis separates analytes according to their electrophoretic mobility. The separation depends on the analytes' charge and size. Basically, CE consists of a sample at the injection side, a small capillary and a detector at the end. A high-voltage field is applied to the capillary and the analytes move in a buffer solution through the capillary.

The analytes are carried towards the cathode by the electroosmotic flow of the buffer solution. Even negatively charged analytes will reach the cathode eventually, because the electroosmotic flow of the buffer is stronger than the electroosmotic mobility of the analytes towards the anode. The first analytes that will reach the cathode are the small, multiply positively charged and the last ones will be the small, multiply negatively charged analytes (Kleparnik & Bocek, 2007).

2.4.3. Quantitative PCR

Quantitative PCR (qPCR) has the same underlying principle as classic PCR. But the classic PCR is an end-point method, where the result is observed after the PCR run. At this point nothing can be said about the initial amount of template. In qPCR the amount of amplicon is measured after each cycle. In order to achieve this a fluorescent dye has to be used. The amount of fluorescence is directly related to the amount of amplicon at the end of each cycle. An ideal PCR run has a sigmoidal curve. The beginning of the curve can not be measured since the fluorescence is lost in background fluorescence, but when a sufficient amount of products are amplified the fluorescence is higher than the background. The curve enters an exponential phase, where – theoretically – the amount of products is doubled in each cycle. Towards the end of the process a linear and a plateau phase are reached, when the nucleotides have been consumed and the produced amplicons are in

competition with the primers (Mackay et al., 2002, Heid et al., 1996, Higuchi et al., 1993).

Several calculation methods have been developed for the enumeration of genes by qPCR. For a review on the different methods see Cikos & Koppel (2009). The most common method is the C_t-method, where a threshold is defined in the exponential phase of the amplification curve. The cycle at which the curves of the amplification cross the threshold is called C_t-value. It is directly correlated with the initial amount of template. The more template existed in the beginning the faster amplification crosses the threshold. Two ways of quantification are used in qPCR which are discussed in the following.

Relative quantification compares the amount of target sequence to an endogenous control or to a related matrix. It provides the amount of templates in relation to another sample or control (Pfaffl & Hageleit, 2001).

Absolute quantification needs a standard with a defined number of gene copies. By creating a standard curve the cycle threshold (C_t) values can be compared to the C_t-values of the template and a copy number can be assigned (Bach et al., 2003). In contrast to relative quantification, it is possible to compare data from different assays and laboratories obtained by absolute quantification (Mackay, 2004).

For quantitative PCR (or real-time PCR) fluorescent markers are introduced which bind to the amplified products. Several types of compounds for amplicon detection have been developed.

SybrGreen is a dye which intercalates into the minor groove of DNA double strands. Thus, it unspecifically marks all double-stranded DNA, which makes additional confirmation necessary that the intended target was actually amplified. This may be achieved for example by melting-curve analysis.

TaqMan probes are oligonucleotides which are labeled with a fluorophor at one end and a quencher at the other end. Due to the close proximity Förster resonance energy transfer (FRET) occurs and results in quenching of the fluorescence of the fluorophor. During PCR amplification the probe is digested by the 5'exonuclease activity of the polymerase and the distance between quencher and fluorophor increases. This leads to higher levels of fluorescence which are directly related to the amount of template.

Hybprobes are two adjacent oligonucleotides where the upstream probe is labeled with a donor fluorophor and the downstream probe with an acceptor fluorophor.

Molecular beacons are designed to create a hairpin structure. The ends are labeled with a fluorophor and a quencher which are in close proximity due to the hairpin structure of the

probe. Upon hybridization of the probe to the template the hairpin is resolved and the distance between fluorophor and quencher increases.

QPCR is sensitive to inhibition by substances which were extracted with the nucleic acids during DNA-Isolation and to other influences that may disturb a successful amplification. Therefore, it is recommended by several authors to include an internal amplification control (IAC) into the assay (Rodriguez-Lazaro et al., 2007, Anonymous, 2002). An IAC is a DNA sequence different from the target sequence which is added to each PCR run and is simultaneously amplified with the target. If no IAC amplification occurs the PCR was inhibited and the results are not reliable. Two major groups of IACs are used.

Heterologous IACs have a completely different sequence than the target gene and have their own primer set which has its own requirements like annealing temperature of the primer pair or $MgCl_2$-concentration. Therefore two different PCR-Assays have to be developed which need the same PCR conditions. This may lead to a reduced efficiency for one or both PCR reactions (Hoorfar et al., 2004).

Homologous IACs (competitive PCR) have the same primer set as the target sequence and preferably the same sequence. They are distinguished from the target by the use of an IAC-specific probe (Malorny et al., 2003, Hoorfar et al., 2004). A homologous IAC has the advantage that it is not necessary to develop a multiplex-PCR which may lead to a loss in sensitivity. As the target sequence and the IAC have to compete for the primers its concentration needs to be optimized (Hoorfar et al., 2004).

2.5. Methods used in this study

One aim of this study was to establish the microbial diversity in vegetable-processing plants.

For a culture-independent approach a 16S rRNA gene clone library was created. This allowed for the detection of bacteria which are either not cultivable or in a VBNC state. Unfortunately, an enumeration is not possible with the molecular clone library technique and it gives no information about the viability of the bacteria.

In contrast to the culture-independent method to measure the microbial diversity, MALDI-TOF MS was tested as a new technique for the identification of bacteria. For MALDI-TOF MS a prior cultivation of the bacteria is necessary. Therefore, an identification of bacteria by MALDI-TOF MS exhibits the same bias as plating-methods, but it also has the same advantages: It allows for a quantification and proves the viability of the bacteria. An added advantage is that the required identification of the bacteria after cultivation is less time-consuming and more accurate than with traditional identification methods.

Isolation of *Arcobacter* by the selective media was unsuccessful as the optimal growth conditions of several strains are unknown. To further ascertain the occurence and genetic diversity of these *Arcobacter* spp. 16S rRNA gene clone libraries were created from samples covering the whole processing line.

A second part of this study concentrated on the evaluation of different methods for the specific detection of pathogens.

Molecular, culture-independent methods are a good choice for a detection system, e.g. a multiplex-PCR, which can distinguish between several species with only one PCR run. Advantages are that PCR is fast and requires small quantities of inexpensive consumables. It can be performed with DNA from many different sample matrices like water, soil, vegetables. Disadvantages are that a quantification is not possible. In order, to assess a health risk it can be important to know how many bacteria are present.

In this study, qPCR-Assays were developed to be able to enumerate *Arcobacter* and *Pectobacterium* as examples of a human- and a phytopathogenic bacterium, respectively. As multiplex-capability of qPCR is very limited, the assays were designed to target several species at once.

In contrast to culture-independent detection methods, MALDI-TOF MS was used to identify *Arcobacter* from vegetable-process water.

3. Material and Methods

Lists for used materials, bacterial strains, media, solutions, kits, restriction enzymes, DNA-marker, plasmids, vectors and oligonucleotides are placed in the appendix.

3.1. Culture conditions

All bacterial strains used in this study were grown in nutrient broth (Carl Roth, Karlsruhe, Germany) under aerobic conditions at 30 °C for 24 – 72 h. In case of *Arcobacter nitrofigilis* 1.5 % NaCl (Carl Roth, Karlsruhe, Germany) was added to the medium as recommended by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

3.2. Sample collection

3.2.1. Carrot-washing plant

Process water was taken from a commercial vegetable washing plant in Sachsen-Anhalt, Germany. At time of sampling, the plant had been processing carrots for several weeks. The plant used fresh drinking water mix with process water from the carrot-washing line. Continuously washing of the produce was performed in a rotating washing drum. Three samples (0.6 l each) were taken directly at the exit valve of the washing drum in December 2006 (denominated as WWd) and from water conduits with fresh tap water (WWs) used for transportation of clean carrots to the packaging area.

3.2.2. Spinach-washing plant

Samples were taken from a spinach processing plant in Brandenburg, Germany, from June 2007 to June 2010. This plant used fresh drinking water for the washing process. The main aim of washing the spinach is the reduction of nitrate in the spinach and to remove the dirt. Depending on the conditions of the field during growth and harvest of the spinach the nitrate content can fluctuate greatly between batches.

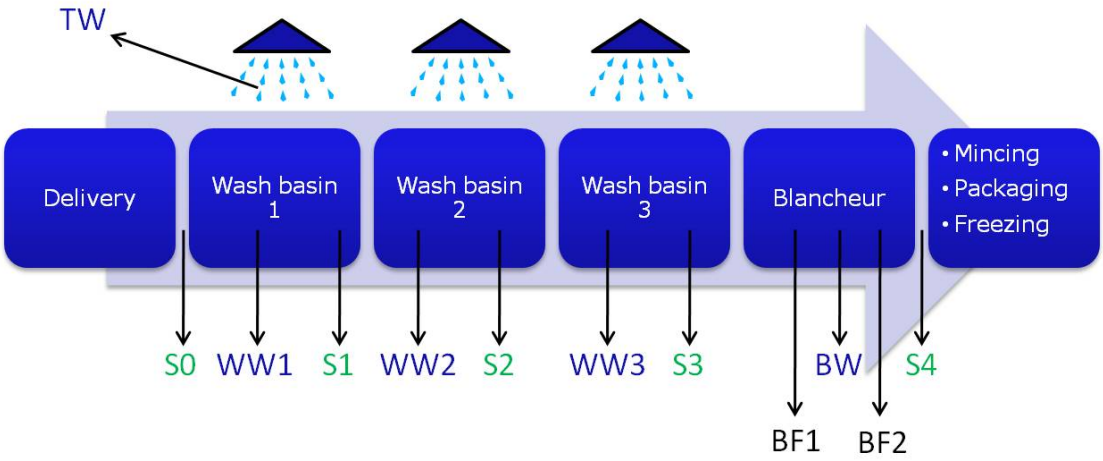

Figure 3.1.: Overview of the spinach-processing line and sample points: uncleaned spinach was delivered to the plant (S0). Samples were taken from tap water (TW) and from water in wash basins 1, 2, and 3 (WW1, WW2, WW3). Samples from partly cleaned spinach is indicated as S1, S2, S3. Water was also sampled from the blancheur (BW). Biofilm from the blancheur was also sampled (BF1, slimy biofilm, BF2, hard biofilm). Spinach was finally sampled before mincing and packaging (S4).

After the spinach is sorted, it is washed in three consecutive basins and each one is operated using fresh water. The washed spinach is blanched before it is sorted by hand, minced, packed and frozen. For an overview of samples taken in the processing-line see Figure 3.1.

In spring 2010, with the beginning of the spinach harvest, the production ran discontinuously since a steady supply of spinach could not be provided. From May 2010 on a continuous production was possible which ran till December with only a short break in August.

Each shift of eight hours starts with filling the wash basins with fresh water. During the shift water quality is checked in terms of nitrate content and only if it is deemed necessary the water is changed in the course of one shift. Every sixth shift the equipment is cleaned. The entire production line is cleaned with high-pressure cleaners filled with water.

The times of sampling are noted in Table 3.1. In the first sampling only wash water samples were taken. In the second sampling the whole processing line was sampled, which became the standard for all following samples. Exceptions were sampling III-05/2008, where no spinach samples were taken, and sampling VIa-04/2010 and VII-06/2010. Sampling VIa-04/2010 took place during the period of time, when the production was stopped during winter. The entire processing line was dried out except for a rest of water (estimated 10 . . . 20 l) in wash basin 2 and 3. Sampling VI-06/2010 took place immediately before a cleaning shift and sampling VII-06/2010 immediately after the cleaning shift before production continued. At that moment no spinach

Table 3.1.: Samples taken at a spinach-processing plant.

Sampling-date	TW	S0	WW1	S1	WW2	S2	WW3	S3	BW	BF1	BF2	S4
I-06/2007	-	-	+	-	+	-	+	-	-	-	-	-
II-11/2007	+	+	+	+	+	+	+	+	+	-	-	+
III-05/2008	+	-	+	-	+	-	+	-	+	-	-	-
IV-07/2009	+	+	+	+	+	+	+	+	+	-	-	+
V-10/2009	+	+	+	+	+	+	+	+	+	-	-	+
VIa-04/2010	-	-	-	-	+	-	+	-	-	-	-	-
VI-06/2010	+	+	+	+	+	+	+	+	+	-	-	+
VII-06/2010	-	-	+	-	+	-	+	-	-	+	+	-

S0 = dirty spinach, TW = tap water, S1-S3 = spinach from wash basins 1 to 3, 1 to 3, S4 = spinach after blanching, BW = water from blancheur, BF1 = slimy biofilm from blancheur, BF2 = hard biofilm from blancheur, plus = sample taken, minus = sample not taken

samples were taken and the blancheur had not been filled with water again. In all samplings, the water sample of the blancheur was taken from an outlet as it was not possible to take samples from inside the blancheur while it was running. After the cleaning shift it was possible to take samples of slime and biofilm from inside the blancheur (samples BF1 and BF2).

Samples were taken with gloves and put in sterile flasks and bags. The beaker used for scooping the water from the basins was sterilized with ethanol (70 %) after every use.

The spinach samples were homogenized in 45 ml water for 2 minutes in a stomacher prior to isolation of the microbial gDNA. The water samples were centrifuged at $4,000\,g$ for 45 minutes and the cell pellets were resuspended in 50 ml aqua dest. prior to DNA isolation in order to concentrate the sample. All samples were stored at $-20\,°C$.

3.3. Isolation and enumeration of bacteria by plate-count

Viable count of samples of carrot wash water Aliquots of samples were diluted and spread on Plate Count Agar (Carl Roth GmbH, Karlsruhe, Germany), MacConkey agar (Carl Roth GmbH, Karlsruhe, Germany) and Chromocult agar (Carl Roth GmbH, Karlsruhe, Germany) in order to determine the viable count of the total bacteria, Gram-negative bacteria, and *Escherichia coli*, respectively. Observed colonies were not further characterized. Additionally, the total particle count was measured using MULTISIZER™ 3 COULTER COUNTER® (Beckman Coulter GmbH, Krefeld, Germany). Plate Count Agar plates and MacConkey agar plates were incubated for two days at 30 °C, and Chromocult agar plates for two days at 37 °C.

Isolation of *Arcobacter* spp. Different methods were used to isolate *Arcobacter* spp. in the samples taken from the spinach-processing plant.

- Isolation of *A. butzleri*, *A. cryaerophilus* and *A. cibarius* according to Houf et al. (2001): aliquots of fresh samples were diluted and enriched in 9 ml of *Arcobacter* selective media (ASB, *Arcobacter* broth + 5 % horse blood, defibrinated + selective supplement 1) and microaerobic (Anaerocult C) incubated at 30 °C. After enrichment 200 µl of solution were plated onto Blood Agar plates and ASB-Agar. Plates were incubated microaerobic up to 7 days at 30 °C.

- Directly with filter method (Baggerman & Koster, 1992): 200 µl of a dilution series of each sample was pipetted onto a Cellulose-Acetate-Filter (0.45 µm pore size) which was placed on a Blood Agar plate. After 30 minutes the filter was removed and the solution was plated. Plates were incubated microaerobic up to 7 days at 30 °C.

- With concentrated samples: 1 ml of concentrated samples (see Section 3.2) were enriched in 9 ml of *Arcobacter* broth with a) SS1 + 5 % horse blood, defibrinated and b) CAT + 5 % horse blood, defibrinated. Enrichment cultures were incubated at 30 °C, microaerobic for 3 days. After enrichment 200 µl of solution were plated onto Blood Agar plates and ASB-Agar. Plates were incubated microaerobic up to 7 days at 30 °C.

- Isolation with marine agar: 100 µl of samples were plated directly on marine agar plates and by the filter method described earlier (Baggerman & Koster, 1992). Plates were incubated at 30 °C microaerobic for 14 days.

- Direct plating: aliquots of samples were diluted and directly plated onto ASB-Agar plates (Houf et al., 2001). Plates were incubated microaerobic up to 7 days at 30 °C.

3.4. Extraction of microbial gDNA

Isolation of gDNA from samples A modified protocol based on Rheims & Stackebrandt (1999) was applied for the extraction and subsequent purification of genomic DNA (gDNA). Total gDNA was extracted from 500 µl of resuspended cells. 10 . . . 15 mg PVPP and 15 µl lysozyme (10 mg/l) were added. After incubation at 37 °C for 60 minutes, 15 µl proteinase K (1 % w/v), 60 µl SDS (10 % w/v) and 60 µl $CaCl_2$ (10 mmol/l) were added and incubated at 65 °C for 45 minutes. The samples were centrifuged at 7,500 g for 10 minutes.

NaCl and CTAB (Murray & Thompson, 1980) were added to the supernatant to final concentrations of 0.7 M and 2 % (w/v), respectively. The solution was incubated for 20 min at 65 °C. Two extraction steps were performed by adding equal volumes of a chloroform-isopentylalcohol mixture (24:1 v/v). The genomic DNA was precipitated by adding 0.25 volumes sodium-acetate (3 mol/l) and one volume isopropanol. The precipitated DNA was recovered by centrifugation

at 20,800 g for 10 min, washed twice with 70 % (v/v) ethanol, resuspended in 10 mM Tris/HCl buffer (pH 8.0) and stored at 4 °C. The quality and quantity of purified DNA was estimated visually by gel electrophoresis. A more precise determination of the amount of genomic DNA was reached by quantification with the fluorescent nucleic acid stain PicoGreen (Quant-iTTM 4 PicoGreen dsDNA Assay Kit, Invitrogen, USA) on a NanoDrop ND-3300 fluorospectrometer (NanoDrop Technologies, Wilmington, USA) (see Section 3.5).

The gDNA of sample WWd and WWs from the carrot-washing plant and the samples from sampling I-06/2007 and II-11/2007 of the spinach-processing plant showed inhibition of the PCR amplification caused by contamination of gDNA with humic acids and other compounds. The gDNAs of these samplings (except TW) were embedded in 1 volume low-melting point agarose (Biozym, Hess. Oldendorf, Germany) and washed twice in TE buffer for 5 h each (Moreira, 1998) to remove the inhibitors.

Isolation of gDNA from pure cultures The gDNA of reference species used for positive controls and test of specificity of primer pairs was isolated according to Pospiech & Neumann (1995) and solved in 50 µl aqua dest.

3.5. Quantification of DNA with a NanoDrop ND-3300 fluorospectrometer

Plasmid-DNA and gDNA was quantified with a NanoDrop ND-3300 fluorospectrometer using the fluorescent dye PicoGreen. The fluorescence of PicoGreen greatly increases after binding to double-stranded DNA. The dye was excited at 470 nm and the emissions were measured at 525 ± 20 nm. The sensitivity of this method is approximately 2 pg and the linear range extends from $1\,\mathrm{ng} * \mathrm{ml}^{-1}$ to $1\,\mathrm{mg} * \mathrm{ml}^{-1}$. A standard has to be prepared which consists of serial dilutions of calf thymus DNA. The standard dilutions and the quantification was carried out according to the manufacturer's guidelines.

3.6. Agarose gel electrophoresis

Agarose gels was prepared with 1.2 % agarose in 1 x TAE-Buffer and $3 \cdot 10^{-7}\mathrm{g} * \mathrm{ml}^{-1}$ ethidium bromide. As marker 3 µg of λ-DNA/*Eco*RI + *Hin*dIII, co-digest, was used. Amplified rDNA restriction analysis (ARDRA) and analysis of multiplex-PCR-Assays made it necessary to separate fragments smaller than 500 bp. Therefore, 3.5 % metaphor-agarose gels were prepared with 1 x TAE-Buffer (for multiplex-PCRs with 1 x TBE-Buffer) and $3 \cdot 10^{-7}\mathrm{g} * \mathrm{ml}^{-1}$ ethidium bromide. The DNA was run with 2 µg of pUK19 DNA/*Msp*I (*Hpa*II)-marker.

3.7. Multiplex-PCR and capillary electrophoresis

PCR conditions for the mPCR with multiplex primer set A (see Table C.3) were as follows:

Table 3.2.: Program of multiplex-PCR-Assay.

Step	Time	Temperature	Repetition
2	5 min	95 °C	1 x
3	30 s	95°C	
4	60 s	55°C	40x
5	60 s	72°C	
6	7 min	72°C	1 x

Steps (2) to (4) were repeated 40 times. Each reaction contained 7 µl DNA_{mix} with $1\,ng * \mu l^{-1}$ of each gDNA, 0.5 µM of each primer, 4 mM $MgCl_2$, 200 µM dNTP mix, 0.5 U *Taq* DNA Polymerase and 1 x *Taq* buffer. During a second PCR, the PCR products from the first PCR were labeled with a universal primer pair which is specific for the tag that had been attached to the individual primer. The universal forward primer is labeled with the fluorescent dye Cy-5. PCR conditions for both PCRs were the same. As template 1 µl of PCR products from the first PCR were used. Amplification followed the program given in Table 3.2. For capillary electrophoresis Cy-5-labeled PCR products were diluted 1:100 in water and 0.5 µl was added to 29 µl sample loading solution (SLS) along with 0.5 µl DNA size standard-600. PCR product sizes were determined using the GenomLab GeXPs software and were compared to the expected PCR product sizes.

For a multiplex-PCR with primer set B $0.6\,ng * \mu l^{-1}$ of each gDNA were used (see Table C.4). All primers were used in concentrations of 0.5 µM except the primer pairs for *A. butzleri* (#502/#503) and *A. marinus* (#495/#496), which were used in a concentration of 1.5 µM each. All other conditions were as described above.

3.8. Construction of 16 rRNA gene clone libraries

3.8.1. Initial 16S rRNA gene-specific PCR

Universal bacterial clone library A fragment of approximately 1,500 bp of the bacterial 16S rRNA gene was amplified from gDNA by PCR using the following primers: 16Sfor (27F) 5'-AGA GTT TGA TCM TGG CTC AG -3' and 16Srev (1492R) 5'- TAC GGY TAC CTT GTT ACG ACT T -3' (Lane, 1991). The PCR conditions were selected as follows:

Table 3.3.: Program of PCR-Assay.

Step	Time	Temperature	Repetition
2	5 min	95 °C	1 x
3	30 s	95 °C	
4	60 s	57 °C	24x
5	60 s	72 °C	
6	7 min	72 °C	1 x

Steps (2) to (4) were repeated 24 times. Approximately 1.5 ng of three pooled DNA preparations were used as templates. The reaction mix was set up with 0.5 U *Taq*-DNA-Polymerase, 2 mM $MgCl_2$, 0.2 mM dNTP-Mix, 0.5 µM of each primer and 1 x PCR buffer to a final volume of 25 µl. In order to reduce the bias introduced by the PCR, the products of ten single PCR reactions were pooled as recommended by several authors (e. g. (Sekiguchi et al., 1998)).

***Arcobacter*-specific clone libraries** Primers #240 and #241 were used for the *Arcobacter*-specific clone libraries. The PCR conditions were as described above. The annealing temperature was set to 58 °C. The products of four single PCR reactions were pooled for the subsequent cloning procedures.

3.8.2. Cloning and amplified rDNA restriction analysis (ARDRA)

The 16S rRNA gene amplicons were cloned into the pGEM-T plasmid (Promega, Mannheim, Germany) via TA cloning and transformed into JM109 competent cells (Promega) according to manufacturer's guidelines. The library was tested for positive recombinant plasmids by double restriction. 1 µl of each plasmid was digested with 1 U *Nco*I and 1 U *Sal*I and incubated for 3 h at 37 °C.

ARDRA of positive recombinant plasmids was performed as published by Klocke et al. (2007). The clones for the universal 16S rRNA gene clone library were digested with *Bsu*RI and *Hin*6I. The *Arcobacter* clones of the 16S rRNA gene library were not distinguishable by digest with the used enzymes, therefore additionally *Rsa*I was used for the construction of ARDRA patterns. Individual ARDRA patterns were used as operational taxonomic units (OTUs), each representing a distinct group of microorganisms. For each OTU, one 16S rRNA gene nucleotide sequence was determined (MWG Biotech, Ebersberg, Germany).

3.8.3. Sequencing and phylogenetic analysis

Nucleotide sequences were examined for possible chimeric artifacts using the software tool MALLARD (Ashelford et al., 2006). Reference sequences were obtained from GenBank, National

Center for Biotechnology Information (NCBI-Database), RefSeq (Pruitt & Maglott, 2007) and SILVA (Pruesse et al., 2007) databases. Phylogenetic trees of OTU sequences and reference sequences from GenBank were constructed with the MEGA 4.0 software (Tamura et al., 2007) and ARB (Ludwig et al., 2004). For alignment and taxonomic allocation of 16S rRNA sequences the following parameters were used: ClustalW 1.6 algorithm (Thompson et al., 1994) with gap opening penalty 20 and gap extension penalty 6.66. Phylogenetic consensus trees were constructed using the Neighbor Joining algorithm (Saitou & Nei, 1987) and the Minimum Evolution algorithm with the Kimura-2-Parameter (Kimura, 1980) as distance correction model and a bootstrap resampling analysis for 1,000 replicates (Felsenstein, 1985). Additionally, trees were constructed with the Maximum Parsimony algorithm (Kolaczkowski & Thornton, 2004). The p-distance expressed as

$$1-p$$

converted to percent provides the similarity of two 16S rRNA gene sequences. P – distances were calculated for the 16S rRNA gene clone library constructed with the universal 16S rRNA gene primer pair on the basis of 1,329 residues and for the *Arcobacter*-specific clone libraries on the basis of 1,363 residues using the ARB software.

The diversity within the 16S rRNA gene libraries was analyzed by rarefaction analysis applying the software Analytic rarefaction (Holland). The rarefaction method evaluates how the species number in a sample changes with the number of individuals (Hughes et al., 2001) and reflects the OTU richness of a clone library. Additionally, the OTU richness was estimated with the Chao-I-richness estimator (Chao, 1987). The Chao-I-index estimates the richness of species, which appeared either only with one or with two individuals. In order to describe the uniformity of the distribution of the individuals on the number of OTU, the evenness $e^{\frac{H}{S}}$ was calculated, where H is the Shannon index and S the total number of clones. The coverage of clone libraries was determined with the formula of Good (Good, 1953),

$$(1-\frac{n}{N})\cdot 100$$

with n as the number of phylotypes (OTU) represented by one single clone and N as the total number of clones.

To estimate the diversity of bacteria the Shannon index H (Wang et al., 2005) was calculated as

$$H=\sum \frac{n_i}{n}\ln\frac{n_i}{n}$$

where n_i is the number of individuals of taxon i and n the total number of organisms of all species. This index gives the proportional abundance of species and reacts sensitively to rare species.

Table 3.4.: Plasmids and vectors used in this study

Plasmid	Primer	Vector	Insert	Manufacturer
-	-	pUC19	-	Fermentas GmbH, St. Leon-Rot
p13748	16Sfor/16Srev	pGEM-T	16S rRNA gene *Arcobacter* sp.	This study
p17856	# 346/# 347	pGEM-T	23S rRNA gene *A. cryaerophilus*	This study
p17868	# 238/# 239	pGEM-T	*rpo*B gene *A. butzleri*	This study
p19306	#436/#433	pGEM-T	23S rRNA gene *A. cryaerophilus*+ pUK-fragment	This study
p17685	#336/#337	pGEM-T	*mdh*-gene of *Pectobacterium atrosepticum*	This study

Simpson‘s index of diversity was calculated as

$$1 - \sum \left(\left(\frac{n_i}{n} \right)^2 \right).$$

It ranges from 0 (representing no diversity) to 1 (representing infinite diversity). Simpson’s index of diversity is more sensitive to abundant species in a sample.

Shannon’s diversity index, Simpson’s index of diversity and evenness were calculated with the software PAST v1.72 (Hammer et al., 2001). EstimateS v8.0.0. (EstimateS) was used to calculate the Chao-I-richness estimator. All nucleotide sequences of the bacterial 16S rRNA gene clone library were deposited at the NCBI GenBank under the accession numbers FJ535114 - FJ535236 and sequences of the *Arcobacter*-specific 16S rRNA gene clone library were deposited under the accession numbers JQ845747- JQ845806.

3.9. Development of qPCR-Assays

3.9.1. Construction of standards

For absolute quantification with qPCR it is necessary to use standards in known amounts. The standard is detected in the same way as the target sequence.

Standards were made by inserting a PCR-Product containing the target region of the qPCR-Assay into the pGEM-T vector. Table 3.4 shows which primer pairs were used to create the fragments for the standard plasmids. Fragments were purified with the QIAquick PCR Purification

Kit, ligated into the pGEM-T vector and transferred into JM109 High Efficiency Competent Cells according to manufacturer's guidelines. The success of the cloning was tested by spreading the *E. coli* cells onto LB-agar plates complemented with Ampicillin $50\,\mathrm{ng} * \mathrm{ml}^{-1}$, X-Galactose and Isopropanolthiogalactosid (IPTG). IPTG induces the lac operon of the vector, and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) is important for the blue or white color of the colonies. When the cells multiply with an unmodified plasmid, they are able to produce the enzyme ß-galactosidase. The hydrolysis of colorless X-Gal by the ß-galactosidase result in the insoluble product (5-bromo-4 chloroindole) which has a bright blue color. White colonies indicate successful insertion since the insert destroys the ability of the cells to hydrolyze X-Gal. After incubation of the plates for 16 h at 37 °C several white cells were picked and grown in 4.5 ml of LB-broth containing Ampicillin and incubated for 16 h at 37 °C using a horizontal shaker TH15. Plasmids were extracted with the Machery and Nagel Nucleospin Kit according to manufacturer's guidelines. Plasmid DNA was digested with *Nco*I and *Sal*I as described above (see Section 3.8.2) and the fragments visualized on an 1.2 % agarose gel. Additionally, the plasmid was sequenced to ensure that the correct insert was cloned. One positive plasmid was retransformed in order to produce more DNA material and ensure the consistency of the standard. Ten colonies were picked and the contained plasmid was isolated. The DNA was pooled, digested with 2 U of *Sca*I for 16 h at 37 °C, and the digestion stopped by incubation at 80 °C for 20 minutes. The linearized plasmid was purified with the QIAquick PCR Purification Kit.

The concentration of the standard plasmid which is determined using the NanoDrop ND-3300 UV spectrophotometer is needed for the calculation of the copy numbers of the standard. The following formula is used for the calculation:

$$\frac{C \cdot N_{\mathrm{A}}}{N \cdot M}$$

in which N is the number of base pairs, C is the concentration of the DNA, N_{A} is the Avogadro constant $6.02 \cdot 10^{23}\,\mathrm{bp\,mol}^{-1}$ and M is the average molecular weight of a base pair ($660\,\mathrm{g/mol}$).

3.9.2. Construction of an internal amplification control

For the construction of the internal amplification control (IAC) a plasmid had to be created which contains the primer sites and as much of the target sequence as possible, but is different in the probe binding site. In order to achieve this, the standard plasmid which contains the 23S rRNA gene of *A. cryaerophilus* was used and an artificial DNA-Fragment was inserted by PCR which serves as the probe binding site of the IAC (see Figure 3.2).

A sequence of the artificial vector pUC19 was selected as probe binding site of the IAC. Two primers were designed. Primer #442 is a reverse primer, its 3'-end is specific to the standard

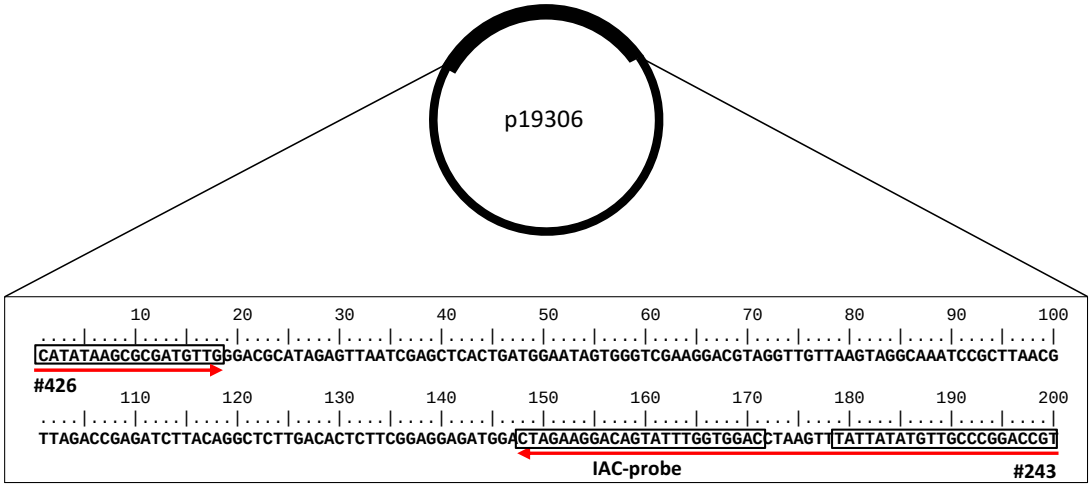

Figure 3.2.: Sequence of the plasmid used as IAC with the primer sites for primer #432 and #433 and the probe binding site for the IAC probe.

plasmid p17856 and targets the region just before the probe binding site of the target. The 5'-end of #442 consists of part of the pUC19-sequence. Primer #443 is a forward primer. Its 3'-end is specific to the other side of the probe binding site of the target and its 5'-end also contains part of the pUC19-sequence in such a way that there is an overlap between the sequences of primer #442 and #443. In a first PCR two fragments were amplified: A fragment of 176 bp was amplified with primers #432 and #442 and p17856 as template. A fragment of 49 bp was produced with primers #443 and #433. It was not necessary to add a template to this PCR as the two primer sequences had an overlap and in fact produced the desired fragment. In a third PCR both fragments were used as templates and primers #432 and #433 were added which amplified the complete 200 bp fragment of the IAC. The product was purified with the QIAquick PCR Purification Kit and treated like the standard plasmids as was described above (see Section 3.9.1). The resulting IAC plasmid was designated as p19306 and sequenced.

3.9.3. Evaluation of DNA-isolation and PCR-amplification with spiking experiments

Spiking experiments were conducted in order to evaluate the efficiency of the DNA-Isolation and the subsequent qPCR-Amplification. A defined amount of cells was added to the samples of the spinach-processing plant before the gDNA was isolated. The isolation procedure was then conducted in parallel to the isolation procedure of the original samples. Pure cultures of *A. butzleri* and *A. cryaerophilus* were grown and the cells were measured with the MULTISIZER™ 3 COULTER COUNTER®. *Arcobacter butzleri* cells were diluted to a concentration of 10^9 cells ml^{-1}

and 1 ml was added to samples of sampling IV-07/2009 and V-10/2009. *Arcobacter cryaerophilus* cells were diluted to a concentration of 10^8 cells ml^{-1} and 1 ml was added to all samples of samplings VIa-04/2010, VI-06/2010, and VII-06/2010. In order to evaluate if the the efficiency was influenced by the spiking 1 ng gDNA of the IV-WW1 sample was spiked with 10^7, 10^8, and 10^9cells of *A. butzleri*. Additionally, the gDNA of 10^7, 10^8, and 10^9cells of *A. butzleri* was isolated and measured with the 16S rRNA gene qPCR-Assay in comparison with the spiked IV-WW1 gDNA.

3.9.4. Calculation of efficiency, limit of detection (LOD) and limit of quantification (LOQ)

The standard threshold method was selected and performed with the ABI PRISM SDS 2.0 software. For each qPCR-Assay a threshold was defined at which the C_t values are taken for subsequent calculation. The threshold should be set in the exponential phase of the amplification curve. The standard curve is constructed by plotting cycles at the threshold (C_t) against the logarithmic values of known amounts of DNA template. A linear regression is calculated, which is used to determine the coefficient of determination (R^2), the slope and the intersection with the y-axis. Efficiency (E) of the PCR-run was calculated with the formula

$$E = 10^{-1/Slope} - 1$$

(Cikos & Koppel, 2009). A PCR efficiency of 1 indicates the highest efficiency, where all target molecules double in one PCR cycle, while an efficiency of 0 indicates no amplification. For limit of detection (*LOD*) and limit of quantification (*LOQ*) the formulas

$$LOD = Intercept + 3\frac{SD}{Slope}$$

$$LOQ = Intercept + 10\frac{SD}{Slope}$$

were used (Mocak et al., 1997). The *LOD* is defined as the lowest signal which can be distinguished from the background fluorescence and *LOQ* is the C_t value which has the lowest fluorescence signal which does not originate from an no-template control (NTC) and which is quantifiable (McNaught & Wilkinson, 1997).

3.9.5. Conditions for qPCR-Assays

The ABI 7300 Real Time PCR System (Applied Biosystems, Darmstadt, Germany) was used to conduct all qPCRs in this study. The rhodamine derivative ROX was used as a passive reference

to normalize the fluorescence. It was contained in every mastermix used in this study.

For each qPCR 1x Mastermix was used (either SybrGreen or TaqMan-Mastermix) with 1 ng of template and the optimal primer concentration. For TaqMan-Assays 250 nM of probe and 10^9copies-IAC plasmid was added. The program was as follows:

Table 3.5.: Program of qPCR-assays.

Step	Time	Temperature	Repetition
1	2 min	50 °C	1 x
2	10 min	95 °C	1 x
3	15 s	95 °C	
4	30 s	57 °C	40x
5	60 s	60 °C	
6*	melting-curve analysis		

*in case of application with SybrGreen as dye

3.10. Analysis by MALDI-TOF MS

For an identification of bacteria by MALDI-TOF MS, aliquots of samples from V-10/2009 were plated onto Plate Count Agar and Blood Agar. Grown colonies were transferred to new plates to ensure a sufficient amount of cell material for MALDI-TOF MS and sequencing. Colonies for MALDI-TOF MS should not be older than $2-3$ days. The MALDI-TOF MS was conducted with the Voyager DE Pro (Applied Biosystems, Forster, USA) in cooperation with the Anhalt University of Applied Sciences, Bernburg. Each colony was picked, smeared on a MALDI-plate and covered with DHB. Results were analyzed with the software provided by the manufacturer and the SARAMIS database (Anagnostek, Potsdam, Germany). It provides comparisons of sample spectra with superspectra of all taxonomic levels. The SARAMIS database is focused on clinically relevant species. A cluster analysis was performed with all spectra obtained in this study. Spectra that had more than 50 % similarity were considered to pertain to a single OTU (Munoz et al., 2011). A colony was chosen for each OTU and a fragment of approximately 1,500 bp of the bacterial 16S rRNA gene was amplified by PCR using the primer pair 16Sfor (27F) and 16Srev (1492R) (Lane, 1991). PCR-Products were purified with the QIAquick PCR purification kit, ligated, transformed and sequenced by GATC Biotech AG (Konstanz, Germany) as described above (3.8). Sequences were checked for chimeric sequences with the software MALLARD (Ashelford et al., 2006) and used in phylogenetic analyses with the ARB software (Ludwig et al., 2004). Statistical analyses were conducted as described above (see Section 3.8.3).

Principal component analysis was done with the R! Excel Software (Neuwirth, 2008). All 16S rRNA gene sequences of bacteria analysed by MALDI-TOF MS were deposited at the NCBI GenBank under accession numbers JQ845807- JQ845877.

4. Results and Discussion

The aim of this study was to investigate the microbial community associated with post harvest processing and to develop and evaluate detection methods for human- and phytopathogens especially associated with vegetable-processing. An overview of the conducted experiments is presented in Figure 4.1.

In the first part of this study molecular methods were used to investigate samples from a carrot-processing plant with the aim to detect bacterial species which before had not been identified in association with vegetable-processing by conventional microbiological methods. Genus-specific PCRs were developed for the further evaluation of samples from vegetable-processing facilities. These PCR-Assays were applied to several samples from vegetable-processing plants and used to construct 16S rRNA gene clone libraries specific for *Arcobacter*. Through the analysis of the genetic diversity of *Arcobacter* it was possible to ascertain which species were prevalent in the process line. It was also determined whether these species are pathogenic and whether vegetables are contaminated during the washing process. Additionally, it was examined whether *Arcobacter* survives in the plant despite of rigorous cleaning procedures.

In the second part of this study three different detection methods for particular pathogens were investigated. As culture-independent methods a multiplex-PCR was developed which provides the means to distinguish nine pathogenic and non-pathogenic species of *Arcobacter*. Additionally, quantitative genus-specific PCR-Assays were developed to allow enumeration of *Arcobacter* and *Pectobacterium*. MALDI-TOF MS was evaluated as a culture-dependent method for ecologic studies of microbial diversity and as a method for detection of pathogens.

4.1. Microbial diversity of a carrot-processing plant

In order to obtain an insight into the bacterial community, samples were taken from a carrot-washing plant.

4.1.1. Viable-count of water samples from a carrot-processing plant

Aliquots of the samples were plated on selective media in order to determine the viable count of the total bacteria, Gram-negative bacteria, and *Escherichia coli*. Yellow colored colonies grew on

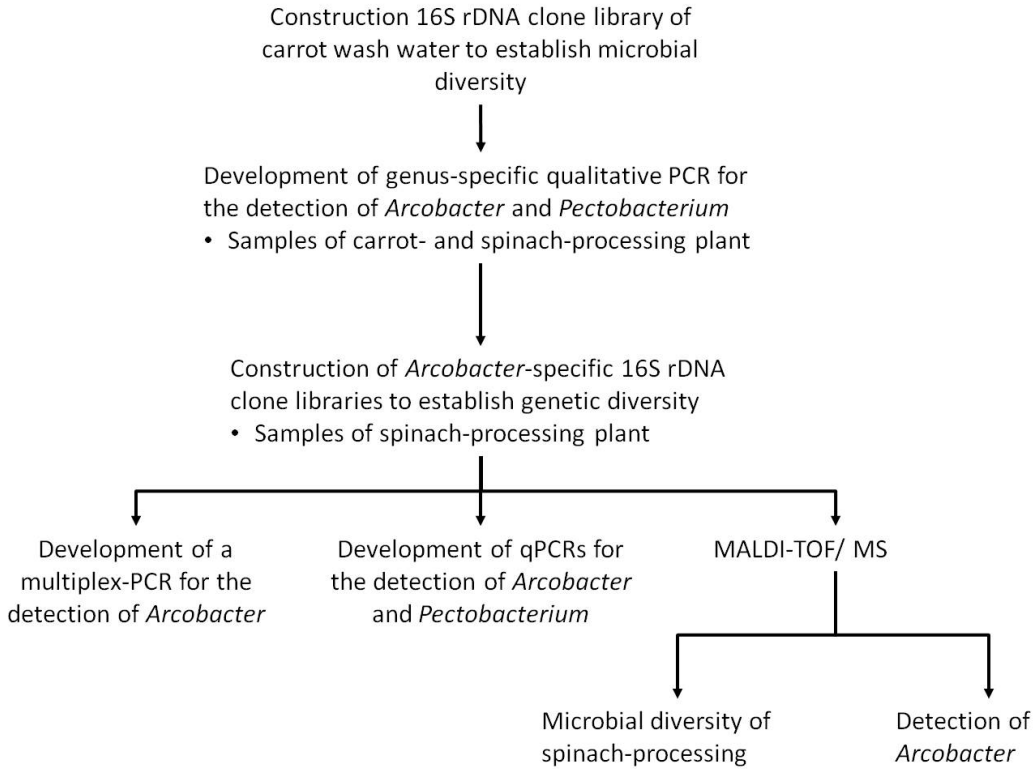

Figure 4.1.: Workflow of experiments conducted in this study. A 16S rRNA gene clone library of wash water was constructed. Then genus-specific primer pairs for qualitative PCR-Assays for the detection of *Arcobacter* and *Pectobacterium* were developed. The *Arcobacter*-specific primer pair was used for the construction of 16S rRNA gene clone libraries. A multiplex-PCR was developed to detect nine *Arcobacter*-species simultaneously. Then *Arcobacter*- and *Pectobacterium*-targeting qPCR-Assays were constructed. Additionally, MALDI-TOF MS was used to establish the diversity of samples from a spinach-processing plant and to detect *Arcobacter* specifically.

Table 4.1.: Viable and total count of bacteria isolated from process water of carrots. Viable-count was established by the plate-count method on Plate Count Agar for "all bacteria" and on MacConkey agar for "Gram-negative bacteria".

	Viable count [cfu ml^{-1}]		Total count [particles ml^{-1}]
Sample	All bacteria	Gram-negative bacteria	All particles*
Wash water	$1.5 \cdot 10^7$	$1.1 \cdot 10^5$	$5.1 \cdot 10^7$
Transportation water	$5.4 \cdot 10^4$	$4.6 \cdot 10^3$	n. d.**

* n. d. = not determined (LOD=10^5 particles ml^{-1})

** with MULTISIZER™ 3 COULTER COUNTER®

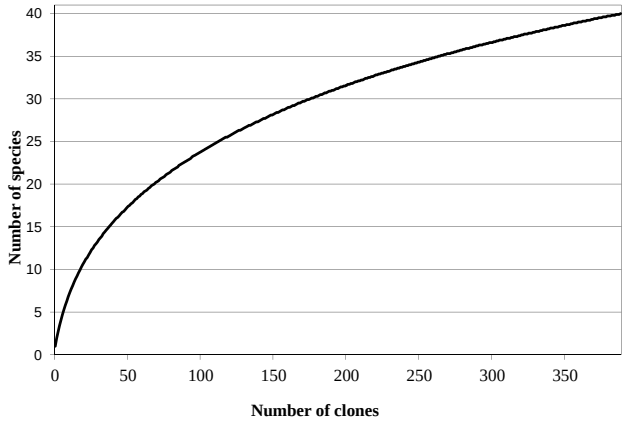

Figure 4.2.: Rarefraction curve of 16S rRNA gene clone library from process water of carrots. The graph shows the number of species over the number of clones in the library. It indicates that not all species prevalent in the samples were isolated.

MacConkey agar plates, indicating the presence of *Salmonella* sp., *Shigella* sp., and *Proteus* sp., while red colored colonies indicated the presence of *Escherichia coli*, *Enterobacter* sp., and *Klebsiella* sp. The grown colonies were not further characterized. Several coliform bacteria such as *Citrobacter freundii* and *Salmonella enteriditis* grew on the Chromocult agar and one *E. coli* colony was isolated. These findings are consistent with other studies establishing the microbial diversity on fresh produce (Beuchat, 1996). The number of particles counted by the MULTISIZER™ 3 COULTER COUNTER® was higher than the viable count numbers (see Table 4.1) obtained using the plate-count method for the wash water sample. The sample from the transportation channel was not suitable for analysis with the MULTISIZER™ 3 COULTER COUNTER® since the number of particles within was too low for a reliable measurement. The limit of detection lies at approximatly 10^5 particles ml^{-1} (Fröhling, A., personal communication).

4.1.2. Results from 16S rRNA gene clone library

With gDNA isolated from the wash water sample a clone library was created using a universal 16S rRNA gene primer pair to establish the microbial diversity of the sample. In total, 427 clones from this library were analyzed by ARDRA. 28 clones representing eight OTUs were removed from further analysis because they were either chimeric or the obtained sequence was of a bad quality. 97 OTUs were identified within the remaining 399 clones. 54 OTUs were represented by singletons. The coverage of the bacterial diversity in the library was estimated to be 87 % using Good´s formula (Good, 1953). The rarefaction curve did not reach saturation suggesting that not

all OTU present in the samples were identified (see Figure 4.2). Calculation of the Chao-I-richness estimator yielded 167 (130 to 241 at 95 % confidence interval) as true number of species in the sample. These estimates also indicate that the sample size of the 16S rRNA gene clone library was too low. Nevertheless, all values support the conclusion that the most prevalent species were identified. The Shannon diversity index and Simpson´s index of diversity and evenness were calculated to be 3.4, 0.9, and 0.3 for the entire 16S rRNA gene clone library, respectively. Using phylogenetic analysis, the sequences were assigned to corresponding reference species for taxonomic classification (see Table 4.2).

Table 4.2.: Distribution of OTUs represented by individual ARDRA fingerprint patterns and the contributing number of clones as detected in the 16S rRNA gene library.

Phylum	Class	Genus	Clones [%]	OTU
Bacteroidetes			14	17
	Bacteroidetes		2	5
		Nubsella sp.	<1	1
		Paludibacter sp.	<1	1
		Parabacteroides sp.	<1	1
		Prevotella sp.	<1	2
	Flavobacteria		12	12
		Chryseobacterium sp.	2	3
		Flavobacterium sp.	11	9
Firmicutes			14	15
	Bacilli		10	10
		Catellicoccus sp.	<1	1
		Lactobacillus sp.	1	3
		Lactococcus sp.	6	1
		Lactovum sp.	<1	1
		Trichococcus pasteurii	<1	2
		Trichococcus sp.	<1	1
		Weissella soli	1	1
	Clostridia		5	5
		Clostridium sp.	4	2
		Veillonellaceae	<1	3
Proteobacteria			71	62
	Alphaproteobacteria		3	10
		Agrobacterium sp.	<1	1
		Caulobacter sp.	<1	1
		Devosia sp.	<1	1
		Rhizobium sp.	<1	2
		Rhodobacter sp.	<1	1
		Rhodopseudomonas sp.	<1	1
		Sphingomonas sp.	1	2
		Stenotrophomonas sp.	<1	1
	Betaproteobacteria		10	22
		Aquaspirillum sp.	<1	1
		Betaproteobacteria	<1	3
		Duganella zoogloeoides	<1	2
		Formivibrio sp.	<1	1
		Janthinobacterium lividum	1	1
		Massilia sp.	<1	1
		Oxalobacteraceae	<1	1
		Polaromonas sp.	<1	1

Phylum	Class	Genus	Clones [%]	OTU
		Propionivibrio sp.	<1	1
		Rhodoferax ferrireducens	4	7
		Undibacterium sp.	1	2
		Zoogloea sp.	<1	1
	Epsilonproteobacteria		11	2
		Arcobacter defluvii	11	1
		Sulfurospirillum sp.	<1	1
	Gammaproteobacteria		47	28
		Acinetobacter sp.	11	5
		Aeromonas sp.	2	2
		Buttiauxella sp.	<1	1
		Enterobacter sp.	<1	1
		Erwinia rhapontici	<1	1
		Enterobacteriaceae	<1	1
		Malikia sp.	<1	2
		Pantoea sp.	<1	1
		Pelomonas sp.	<1	1
		Pseudomonas amygdali	<1	1
		Pseudomonas sp.	3	2
		Rahnella aquatilis	<1	3
		Rheinheimera soli	<1	1
		Tolumonas auensis	26	6
		Yersinia sp.	< 1	2
Fibrobacteres	Fibrobacteria	*Fibrobacter* sp.	<1	1

All the sequences clustered into four major bacterial phyla: *Bacteroidetes, Firmicutes, Fibrobacteres* and *Proteobacteria*. The *Proteobacteria* were represented by 71 % of 399 clones in the 16S rRNA gene library (62 OTUs). All classes except the *Deltaproteobacteria* were detected. The most prevalent class was the *Gammaproteobacteria* with 28 of all OTU being detected in over 47 % of the clones. The most prevalent genus was *Tolumonas* sp. with 26 % of the clones represented by six OTUs. One OTU of this genus was represented by 93 clones of the 16S rRNA gene library. *Tolumonas* is reported as a non-pathogenic, toluene-producing member of the *Aeromonadales*. The classes of *Beta-* and *Epsilonproteobacteria* were represented with 10 % and 11 % of all clones, respectively (*Betaproteobacteria* with 25 OTUs and *Epsilonproteobacteria* with four OTUs). Another genus which includes several known pathogenic species detected in the 16S rRNA gene library was *Acinetobacter* sp. with 11 % of the clones spread over five OTUs. The genus *Acinetobacter* contains certain opportunistic pathogens which are known to cause serious nosocomial infections. 11 % of the clones were assigned to *Flavobacterium*. Some *Flavobacterium* species cause diseases in fish and nosocomial infections in humans. The also potentially pathogenic *Clostridium* sp. and *Pseudomonas* sp. were represented by 5 % and 3 % of the clones, respectively. Overall, the identified microorganisms are mostly typical soil or water-borne bacteria. Surprisingly, *Pectobacterium* was not detected as could have been expected because it is the most common pathogen on carrots and potatoes (Toth et al., 2003).

The genus *Arcobacter* was determined to be the fourth most prevalent genus in the library

with 11 % of all clones represented by two OTUs. The clones ATB-LH-6148, ATB-LH-5950, and ATB-LH-5962 were assigned to the class *Epsilonproteobacteria* based on comparisons with reference sequences obtained from GenBank.

Their sequences showed a 97.8 . . . 98.6 % sequence similarity with *Arcobacter defluvii* that was isolated from wastewater in Spain, suggesting that this *Arcobacter* species is also free-living, waterborne and not epiphytic (see Figure 4.3). In previous studies the presence of certain *Arcobacter* species with pathogenic potential was shown for various meat products, but also for fecal samples (e.g. *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius* (Phillips, 2001)). Recently, González & Ferrús (2011) detected *Arcobacter* spp. on salad.

Several sources of contamination of the wash water with *Arcobacter* are hypothetically possible. Primarily, an introduction through the potable water is conceivable. The examined plant uses tap water to wash the vegetables. Additionally, potable water is used to transport the processed carrots in water filled conduits to the packaging area. *Arcobacter butzleri* was isolated from certain water sources, a drinking water reservoir in Germany (Jacob et al., 1993, 1998), a well-water source in the USA (Rice et al., 1999) and, more recently, in seawater and plankton samples from the Mediterranean Sea (Fera et al., 2004). This suggests that *Arcobacter butzleri* is able to survive in water sources and may be distributed by the water.

Another possible source for *Arcobacter* is the soil adhering to the vegetables. Stampi et al. (1993, 1999) detected *Arcobacter* species in sewage and activated sludge through all stages of treatment and digestion. Therefore, sludge distributed on the field as fertilizer seems a possible mode of transmission. However, the soil attached to the carrots has not been examined separately in this study.

Once introduced into the washing water and the washing process *Arcobacter* may have attached itself to the equipment of the washing plant. Assanta *et al.* (2002) showed that *Arcobacter* has the capacity to attach itself to surfaces of water distribution pipes made from copper, plastic or stainless steel and glass and to survive for longer periods even when the surface had completely dried (Cervenka et al., 2008). The analyzed washing drum is made of stainless steel. So it seems possible that *Arcobacter* colonized the washing drum and was suspended in and continuously mixed with the newly added fresh drinking water. In consequence this may lead to a contamination of the vegetables from one batch to another.

4.2. Detection of *Arcobacter* and *Pectobacterium* by PCR

After *Arcobacter* was detected in the wash water of a carrot-processing plant the question about a general occurence of *Arcobacter* in vegetable-processing plants needed to be addressed. A further examination of the routes of transmission and the occurrence of *Arcobacter* in association with

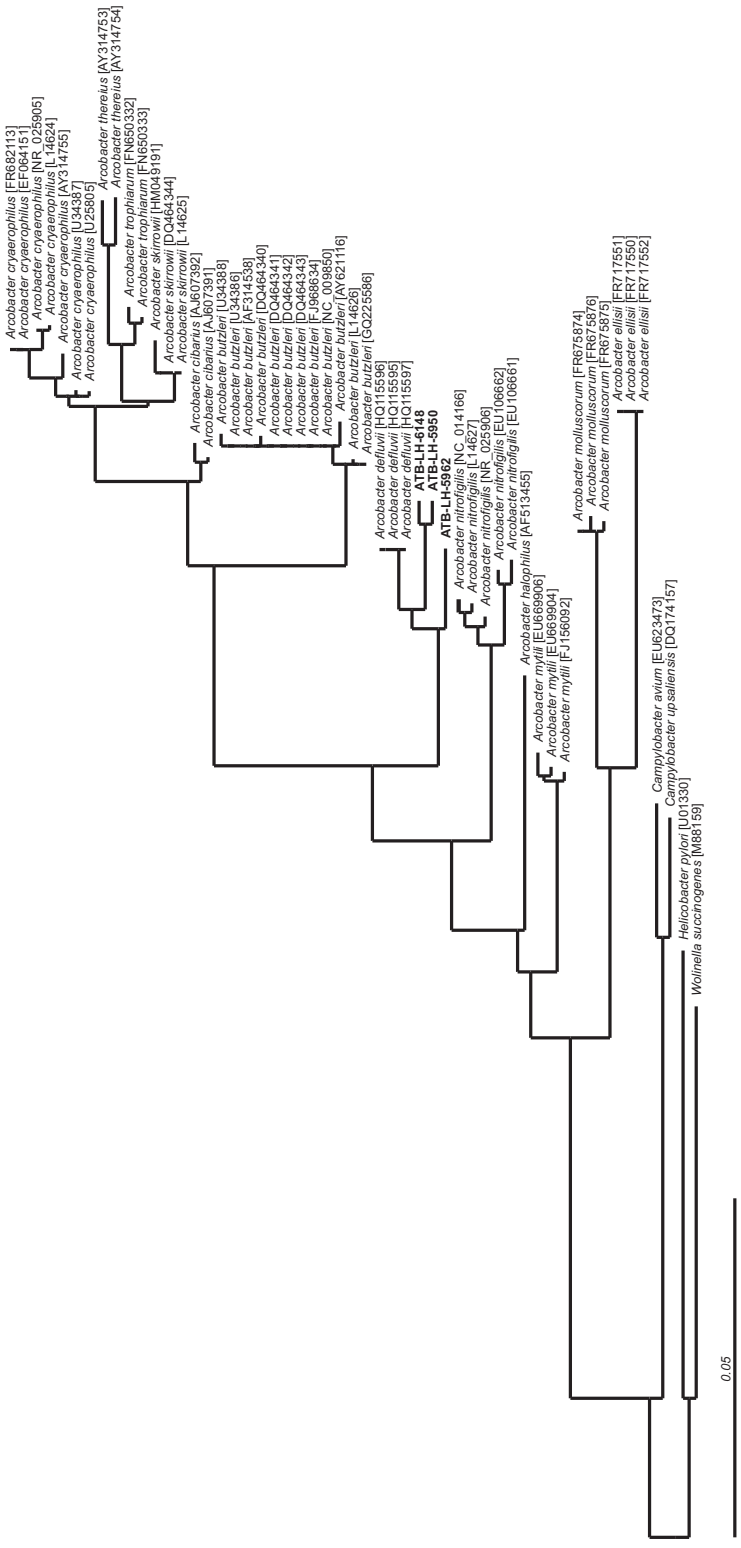

Figure 4.3.: Phylogenetic tree of the determined OTUs among the family *Campylobacteriaceae*. The tree was constructed using the Maximum parsimony algorithm (Kolaczkowski & Thornton, 2004). Members of the family *Helicobacteraceae* were used as outgroup. Numbers in brackets indicate GenBank accession numbers. All OTUs determined in this study are designated with "ATB" and printed in bold. The bar represents 5 % evolutionary distance.

vegetable-processing was necessary in order to perform an evaluation of potential contamination risks. The aim was to screen for *Arcobacter* spp. by the means of PCR, which allows for a specific, reliable and fast detection. Therefore, suitable PCR-Assays from literature were tested and an *Arcobacter*-specific PCR-Assay was developed and used on samples additionally obtained from a spinach-processing plant.

4.2.1. Evaluation of existing PCR-Assays for *Arcobacter*

An extensive search for published PCR-Assays specific for *Arcobacter* spp. revealed that many primer exist, but most of them were specific for *Arcobacter butzleri* only.

Initially, a multiplex-PCR published by (Houf et al., 2000) was used (see Table B.8). According to Houf et al. (2000) the assay distinguishes between *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* and has been used by several authors to detect the selected *Arcobacter* sp. in environmental samples (Atabay et al., 2008, Collado et al., 2008, Fera et al., 2004, Gugliandolo et al., 2008). This assay gives no amplicon for the species *A. cibarius* (Houf, personal communication). Additionally, the *A. skirrowii* primers produced an amplicon with gDNA of *A. nitrofigilis* (data not shown) which was originally used as negative control. Later this was published by Figueras *et al.* (2008). With these restrictions in mind, the multiplex-PCR was performed as described by Houf *et al.* (2000) on gDNA of the wash water and the potable water of the transportation conduits of the carrot processing plant. No PCR product was obtained from any sample (data not shown) suggesting that *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. nitrofigilis* were not detectable either because they were not contained in the samples or the number of cells was below the limit of detection of the assay. Houf et al. (2000) report the detection limit for *A. cryaerophilus* and *A. butzleri* to be $10^3\,\mathrm{cfu\,g^{-1}}$ in a chicken sample and for *A. skirrowii* to be $10^2\,\mathrm{cfu\,g^{-1}}$ in a chicken sample. As no Arcobacter spp. were detected with the multiplex-PCR, a new PCR-Assay was developed which was designed to detect all known *Arcobacter* species including *Arcobacter defluvii* (see Figure 4.4).

4.2.2. Development of a new *Arcobacter*-specific assay

In order to determine the presence or absence of *Arcobacter* in other water samples a new *Arcobacter*-specific primer pair (#240/#241) was developed *in silico* (see Table B.8). They detect most of the sequences deposited in the GenBank database and the sequences established in this study. The optimal annealing temperature was determined by a PCR with gradually increased temperatures to be 58 °C (see Figure 4.5).

The specificity of the primer pair was tested against a panel of *Arcobacter* strains and against *Pectobacterium* strains as negative controls. During the construction of several *Arcobacter*-

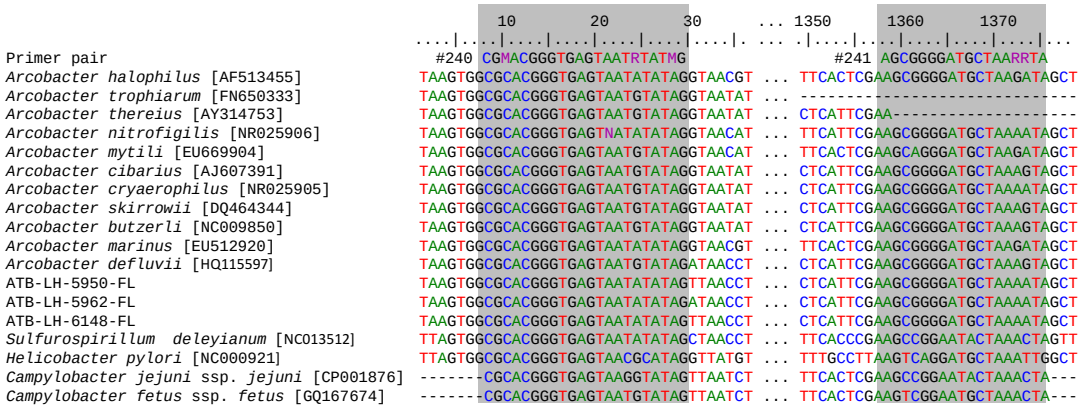

Figure 4.4.: Alignment showing target sequences of *Arcobacter*-specific primer pairs (areas marked in grey). Numbers in brackets indicate accession numbers.

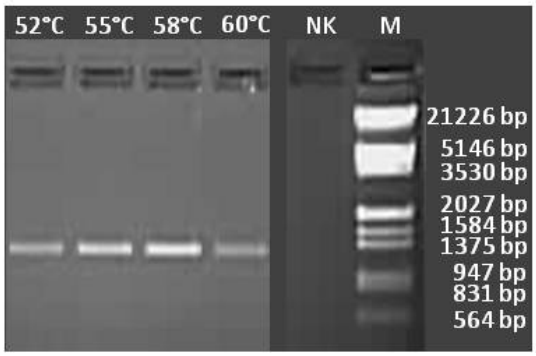

Figure 4.5.: Determination of optimal annealing temperature by gradient PCR with *Arcobacter*-specific primer pair #240/#241. The template in this PCR was gDNA from process water of carrots. Additional columns indicate negative control (NK) and marker (M).

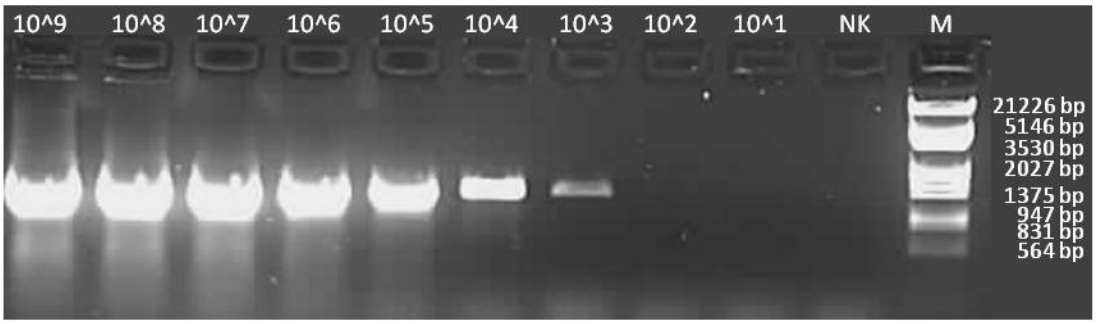

Figure 4.6.: Limit of detection (LOD) of *Arcobacter*-specific assay. Varying numbers of copies of plasmid p13748 containing 16S rRNA gene of *Arcobacter skirrowii* were used ($10^1 - 10^9$copies $*\,\mu l^{-1}$). Additional columns indicate negative control (NK) and marker (M).

specific clone libraries with these primers several environmental samples were screened with the primer pairs and around 200 clones were sequenced. Not once a clone was isolated that did not belong to the *Arcobacter* genus. This proves that the primer pair is highly specific for the tested *Arcobacter* strains. The limit of detection was determined in a PCR with a plasmid containing the 16S rRNA gene of *Arcobacter skirrowii* in decreasing concentration with 10^9 copies to 10^1 copies. It was established at 10^3 copiesμl^{-1} (see Figure 4.6).

4.2.3. Detection of *Arcobacter* in vegetable-processing plants by PCR

First, gDNA from the water samples of the carrot-washing plant was tested with the *Arcobacter*-specific 16S rRNA gene primers. A PCR product was amplified only with the wash water. An analysis of the water in the transportation conduits did not confirm any contamination with *Arcobacter* at the particular moment when the sampling of this study was conducted.

Additional samples were taken from another, a spinach-processing plant. It had a more complex process line than the carrot-processing plant (see Figure 3.1). Therefore, samples were taken from several points and in the course of several years in order to evaluate the distribution of *Arcobacter* in the process line during the washing process and the influence of seasonal changes and disinfection procedures (see Table 3.1).

The results of the screening are shown in Table 4.3 and exemplary in Fig. 4.7 for the sampling III-05/2008.

As can be seen in Table 4.3 the occurrence of *Arcobacter* varies with time and sample location. It was always detectable in wash basin 2. *Arcobacter* is recurrently detectable in the wash water samples, but rarely on spinach samples. In our study *Arcobacter* was detected only once in the blanching water sample or on the clean spinach.

The most contaminated sampling was IV-07/2009, at which time the plant had been contin-

Table 4.3.: Overview over the occurrence of *Arcobacter* in all samples from the spinach-processing plant.

Sampling	TW	S0	WW1	S1	WW2	S2	WW3	S3	BW	S4
I-06/2007	n.a.	n.a.	-	n.a.	+	n.a.	+	n.a.	n.a.	n.a.
II-11/2007	-	-	+	-	+	+	+	-	-	-
III-05/2008	-	n.a.	+	n.a.	+	n.a.	+	n.a.	-	n.a.
IV-07/2009	+	+	+	+	+	+	+	+	+	+
V-10/2009	+	-	-	+	-	+	+	+	+	+

S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching; n.a. = not analyzed.

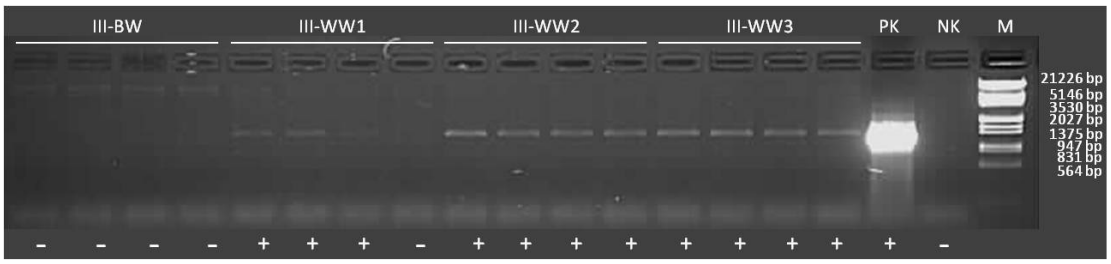

Figure 4.7.: Qualitative PCR applied to samples from III-05/2008. The columns indicate water from the blancheur (III-BW), wash water from basin 1, 2 and 3 (III-WW1, III-WW2, III-WW3). Additional columns indicate plasmid p13748 with 16S rRNA gene of *Arcobacter skirrowii* (PK), negative control (NK)and marker (M).

uously processing spinach for weeks. *Arcobacter* was detectable on the uncleaned spinach, in the tab water source and on all the following samples. These results indicate that in that instance contaminated spinach had been processed, which raised the amount of bacteria, so that *Arcobacter* was detectable in all samples.

It is also possible that cross-contamination occurred. However, cross-contamination during DNA-Isolation and setting up the PCR was ruled out since the DNA-Isolation was repeated several times, while the results did not change and negative controls of the PCR-Runs were always negative. The tab water sample was taken directly at the exit valve above the wash basin. It is conceivable, that *Arcobacter* might be present on the exit valve as all surfaces are wet and the air is rich with aerosols. Results for sampling V-10/2009 are inconsistent with the other results obtained with this PCR-Assay, as the WW2 sample is negative. In all other tests it had been positive. Additionally, the V-TW sample is positive, while the first wash water sample with a positive result for *Arcobacter* is V-WW3. With the exception of the uncleaned spinach all spinach samples are positive. Either V-TW, V-S1 and V-S2 are false positive or V-WW1 and V-WW2 are false negative.

The results do not indicates whether blanching seems to be effective against *Arcobacter*,

because traces of *Arcobacter* could be detected in the water leaving the blancheur and on the blanched spinach. As PCR results do not give any information about the viability of the cells, it is possible that the PCR detects dead cells and blanching is effective. Nevertheless, vegetables that are only washed and not blanched could be delivered contaminated to the consumer.

4.2.4. Evaluation of existing *Pectobacterium* assays

In comparison to the human pathogen *Arcobacter, Pectobacterium* was chosen as an example for a phytopathogen and the samples were tested for its occurrence. Several primer pairs were tested for their specificity to *Pectobacterium carotovorum* ssp. *carotovorum* (see Figure 4.8). In our analysis the primers known from literature were either non-specific or did not work as described in the literature.

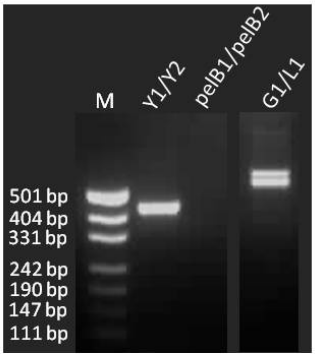

Figure 4.8.: PCR with primer pairs specific for *Pectobacterium carotovorum* ssp. *carotovorum*. The columns indicate marker (M), primer pair Y1/Y2 (Darrasse et al., 1994), primer pair pelB1/pelB2 (Yap et al., 2004), primer pair G1/L1 (Jensen et al., 1993).

Only primer pair Y1/Y2 yielded the desired fragment of 435 bp with gDNA of *P. carotovorum* ssp. *carotovorum* (Darrasse et al., 1994). Primer pair G1/L1 targets the ITS-region of *P. carotovorum* (Jensen et al., 1993). As expected several fragments were amplified, because several ITS-Regions exist, but not all fragments were amplified as expected. The PCR with the primer pair pelB1/ pelB2 (Yap et al., 2004) did not result in the amplification of any fragment. The PCR was repeated with different annealing temperatures and with different $MgCl_2$-concentrations, resulting in amplification of the desired, but also of unspecific fragments (data not shown). The primer pair was used in the original paper on *Pectobacterium carotovorum* isolates, but not on the type strain. The sequence for the *pel*B-gene of the type strain is not deposited in the NCBI database. It could be different to the isolates, so that pelB1/pelB2 do not anneal. Primer pair AFP18/ AFP19 is specific for a fragment of *P. carotovorum* ssp. *carotovorum* [AF046928] (Brouwer et al., 2003). It proved to be highly specific for this subspecies (see Figure 4.9).

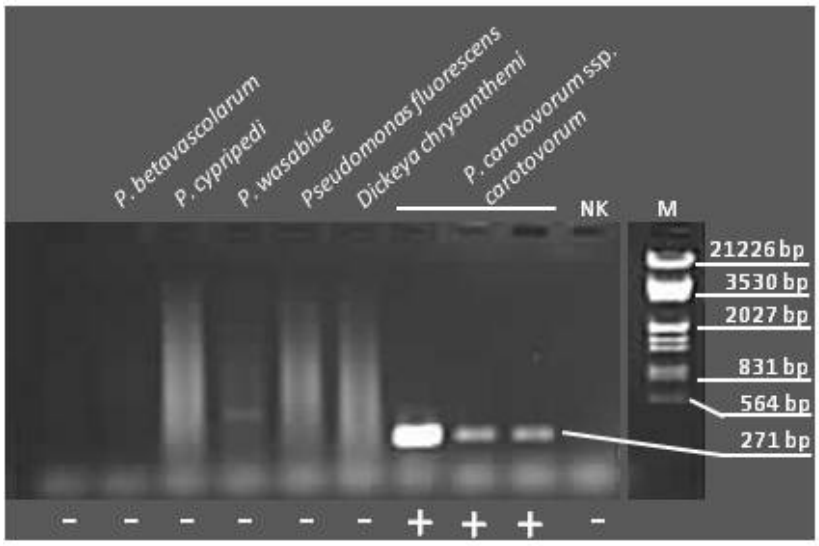

Figure 4.9.: Specificity of primer pair AFP18/ AFP19. Genomic DNA from carrot wash water was used as template. *P. carotovorum* ssp. *carotovorum* = plasmid DNA, gDNA from two different isolations; NK = negative control; M = marker.

For the screening of vegetable-processing plants, it was intended to use an assay which is specific for several *Pectobacterium* species, therefore a new assay was developed.

4.2.5. Development of *Pectobacterium*-specific assays

The aim of the assay development was a genus-specific primer pair for *Pectobacterium*. It was not possible to identify a primer pair based on the 16S rRNA gene of *Pectobacterium* since the 16S rRNA gene sequences were too much conserved to include all *Pectobacterium* species and rule out all other Enterobacteriaceae. Therefore the *pel*B-gene was selected as target gene. The *pel*B-gene encodes for a pectate lyase, which is a virulence factor. They are the main cause for the soft rot symptoms during infection with plant pathogens (Payasi et al., 2009). Targeting a virulence factor is an advantage for the assay because a positive result in PCR would not only show the existence of *Pectobacterium*, but also of a pathogenic species. The sequences of the *pel*B-genes were not conserved within the species, making it difficult to identify primer pairs which would detect all *Pectobacterium* species (see Figure 4.10).

An attempt was made to design a primer pair which amplifies DNA from as many *Pectobacterium* species as possible. Two primer pairs were constructed. The first pair was designated #188/#189, the second one #194/#195 (see Table B.8). The specificity was tested on a panel of

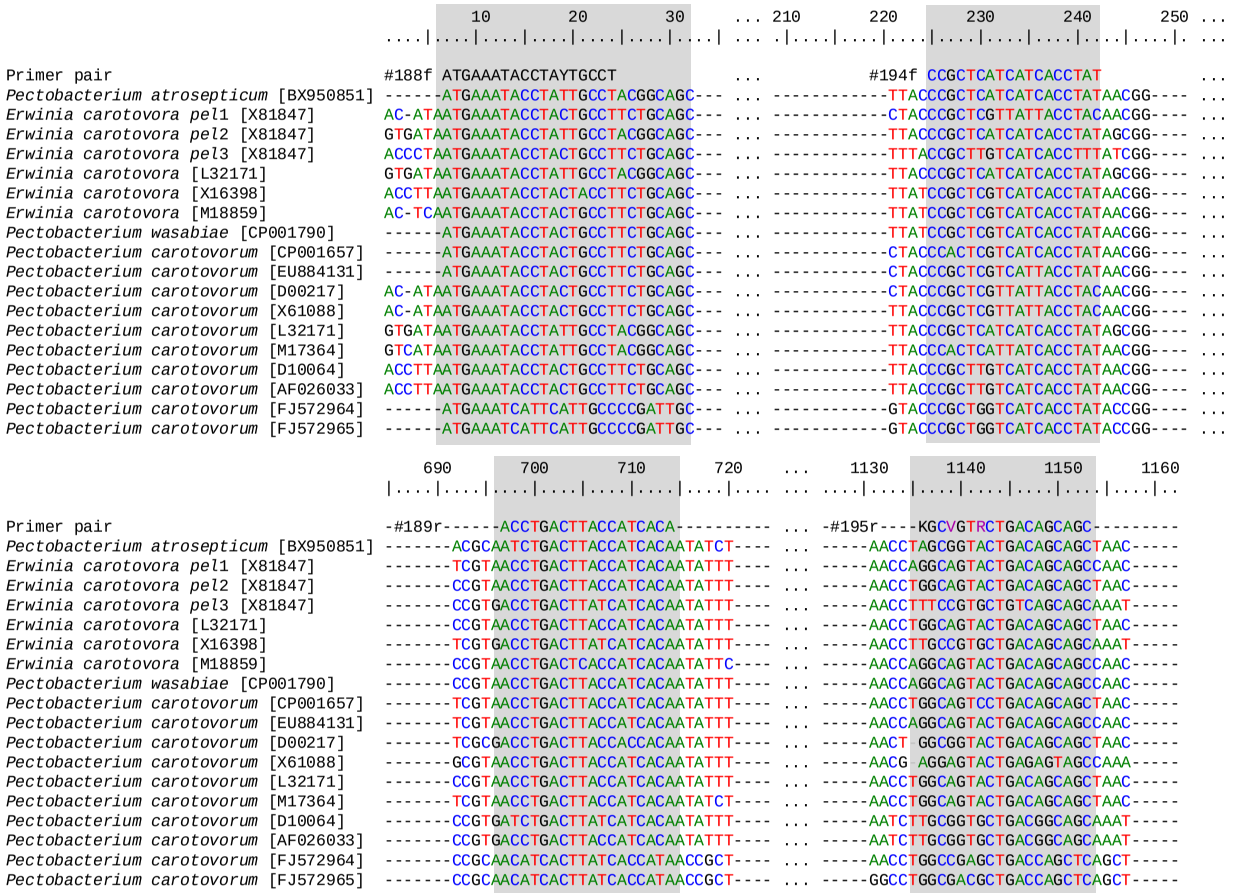

Figure 4.10.: Alignment showing target sequences of *Pectobacterium* primer pairs (areas marked in grey). Numbers in brackets indicate accession numbers.

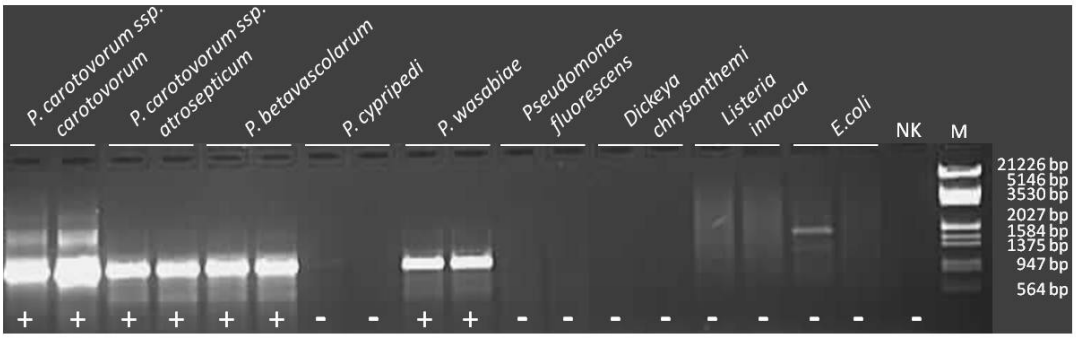

Figure 4.11.: Specificity of primer pair #188/#189. Template = gDNA, NK = negative control; M = marker.

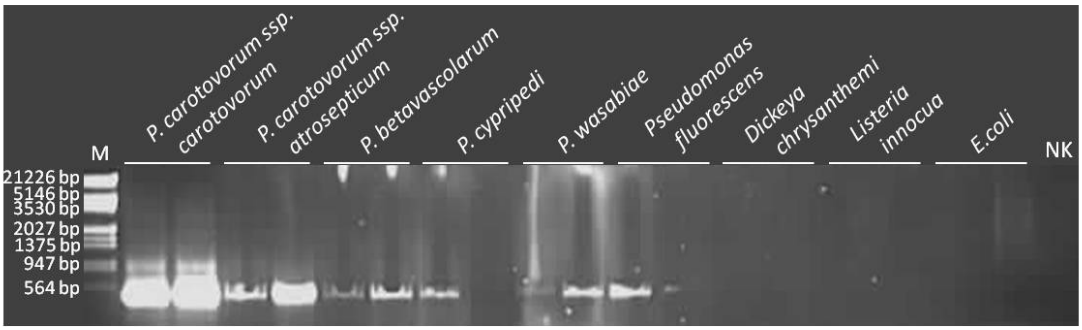

Figure 4.12.: Specificity of primer pair #194/#195. M = marker; Template = gDNA; NK = negative control.

several *Pectobacterium* strains (see Table B.10) and the Gammaproteobacteria *Pseudomonas fluorescens*, *Dickeya chrysanthemi*, *Listeria innocua* and *E. coli* (see Figure 4.11). Unfortunately, an unspecific fragment of *E.coli* was amplified as well. Therefore the primer pair was not used in further experiments.

A test of primer pair #194/#195 showed a specificity for most of the *Pectobacterium* strains, except *Pectobacterium cypripedii* and showed no PCR-products with the non-*Pectobacterium* strains (see Figure 4.12).

Therefore, it was used in the screening of samples of the vegetable-processing plants. The annealing temperature of primer pair #194/#195 was established to be at 60 °C (see Figure C.1).

4.2.6. Detection of *Pectobacterium* by PCR in a spinach-washing plant

The screening of the samples of the carrot and the spinach-processing plant showed heterogeneous results (see Table 4.4). In the carrot-washing plant *Pectobacterium* was not detectable by PCR or selective media at the time of sampling.

Table 4.4.: Overview over the occurrence of *Pectobacterium* ssp. in all samples from the spinach-processing plant as detected by PCR with primer pair #194/#195.

Sampling	TW	S0	WW1	S1	WW2	S2	WW3	S3	BW	S4
I-06/2007	n.a.	n.a.	+	n.a.	+	n.a.	+	n.a.	n.a.	n.a.
II-11/2007	-	-	+	+	+	+	+	-	-	-
III-05/2008	+	n.a.	+	n.a.	+	n.a.	+	n.a.	+	n.a.
IV-07/2009	-	+	+	+	+	+	+	+	+	+
V-10/2009	-	-	-	-	-	-	-	-	-	-

S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching

In the spinach-processing plant *Pectobacterium* was always detected at least in the wash basins, except for sampling V-10/2009, where no *Pectobacterium* was found. For uncleaned spinach only spinach of sampling IV-07/2009 gave a positive result. In general, similarities to the PCR-based detection of *Arcobacter* can be observed: the sampling spinach IV-07/2009 was contaminated most, while the sampling spinach V-10/2009 was contaminated least. For the most part positive results were obtained from the wash basins, similar to the results for *Arcobacter*. In contrast to *Arcobacter*, there seem to be periods of time without *Pectobacterium* contamination in the plant. No deduction could be made, whether spinach was contaminated with *Pectobacterium* during the washing process.

4.3. Genetic diversity of *Arcobacter* in a spinach-processing line

In this study *Arcobacter* was detected in wash water from a carrot-processing plant by construction of a 16S rRNA gene clone library. This was the first time *Arcobacter* had been identified in association with vegetable-processing. The development of an *Arcobacter*-specific PCR-Assay revealed that *Arcobacter* was also detectable in a spinach-processing plant.

The occurrence of similar *Arcobacter* sequences at two different plants processing different kinds of vegetables suggests that *Arcobacter* may be common in vegetable-processing. So far, the route of transmission is unclear. Additionally, the questions must be answered, whether vegetables are contaminated during the washing process and if the detected *Arcobacter* strains

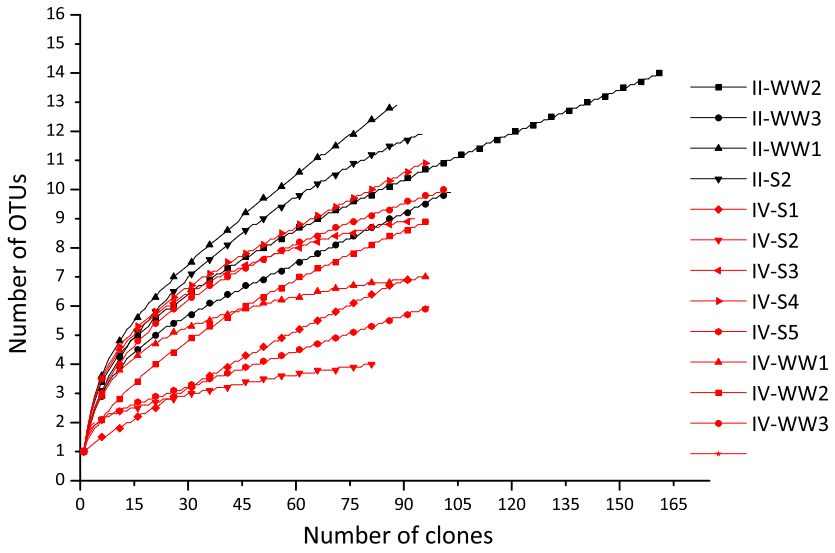

Figure 4.13.: Rarefraction curves of *Arcobacter*-specific 16S rRNA gene clone libraries showing the species richness of the samples. Red indicates samples from IV-07/2009, while samples from II-11/2007 are shown in black. S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching.

belong to the pathogenic species. Since the developed PCR-Assay does not distinguish between the different *Arcobacter* species, several *Arcobacter*-specific 16S rRNA gene clone libraries were constructed. Samples from the spinach-processing plant were used to obtain information on the genetic diversity and the dispersion pattern of *Arcobacter* strains in the processing line.

It is possible, that not only non-pathogenic *Arcobacter* species are prevalent. In order to determine, if a contamination of the product occurs during processing the entire processing line was sampled in 2007 and from each sample containing *Arcobacter* a separate clone library was constructed. The procedure was repeated in 2009 in order to observe if *Arcobacter* persists on the equipment regardless of discontinuation of production in winter and of the disinfection and cleaning procedures in the plant.

4.3.1. Richness, diversity and evenness of constructed 16S rRNA gene clone libraries

The entire spinach-processing line was sampled as described previously (see Section 3.8.3). Samples were checked for the occurrence of *Arcobacter* by *Arcobacter*-specific PCR (see Section 4.2.2). Samples with a positive result were used in the construction of the 16S rRNA gene

clone libraries. The sampling and analysis process was repeated after two years. For each detected OTU, sequences were determined and phylogenetically analyzed.

Table 4.5 shows the isolated clones of each library, the detected OTUs and the numbers of established species.

In 2007 *Arcobacter* was not detectable in all samples, therefore a clone library was not constructed for all of the samples. In 2009 *Arcobacter* was detected in all samples, but only barely in the drinking water (IV-TW) and blancheur water (IV-BW) samples. This is probably the result of a contamination during PCR or sampling (for a discussion see subsection 4.2.3). For the sake of completeness, the results for these samples are included in the analysis, but should be considered with caution, as the low number of clones does not provide a solid analytical base. The rarefaction curves (see Figure 4.13) and the coverage show that the most abundant OTUs were identified in the clone libraries. The coverage ranges from 67 % of the IV-BW-sample to 100 % of the IV-TW-sample. The results for the Chao-I-richness estimator of the libraries from 2009 lie inside the 95 % confidence interval (or little below), but mostly at the lower boundary. This suggests that the most abundant OTUs were identified, but the sample size was to low to identify all OTUs.

The average Shannon diversity index was calculated to be 1.57 ± 0.19 for the samples of 2007, showing that they have similar diversities. The average Shannon diversity index for the samples from 2009 is 1.01 ± 0.44. The diversity in these samples is not as evenly distributed as in the 2007 samples and in comparison to 2007 the diversity is lower. This is supported by the results for evenness, which are low with 0.36 ± 0.04 (2007) and 0.53 ± 0.26 (2009) for all libraries except IV-TW and IV-BW, where the sample size has been too small.

This shows that in all samples OTUs are not evenly distributed, but instead some OTUs are dominant. The samples taken in 2007 have a higher diversity than the samples taken in 2009. However, the sample size was not sufficient to calculate Chao-I-richness properly. This conclusion is supported by the coverage parameter and the fact that the detected OTUs do not lie between the 95 % confidence intervals of the Chao-I-richness estimator.

4.3.2. Comparison of the diversity and dispersion of OTUs of the 16S rRNA gene clone libraries

The phylogenetic analysis revealed that several OTUs represent *Arcobacter* species which had already been described (see Figure 4.14).

In addition to the constructed dendrogram, the sequences were compared to reference sequences in the NCBI database (see Table C.2).

If a sequence had more than 99 % similarity to a reference species it was considered to be a member of said species (Keswani & Whitman, 2001). Several pathogenic species were detected,

Table 4.5.: Numbers of isolated clones, detected OTUs and established *Arcobacter* species of the 16S rRNA gene clone libraries.

	TW	S0	WW1	S1	WW2	S2	WW3	S3	BW	S4
II-11/2007	ND	ND	+	+	+	ND	+	ND	ND	ND
Clones			89	84	162		104			
OTUs			14	13	16		11			
Taxa			7	5	6		6			
Singletons			8	6	9		6			
Chao 1			30 (17/ 98)	19 (14/ 46)	56 (22/ 277)		29 (13/ 138)			
Shannon			1.79	1.48	1.65		1.37			
Evenness			0.4	0.3	0.3		0.4			
Coverage*			91 %	93 %	94 %		94 %			
IV-07/2009	+	+	+	+	+	+	+	+	+	+
Clones	5	92	99	96	98	94	102	97	3	98
OTUs	2	7	8	5	9	9	10	11	2	6
Taxa	2	4	5	3	6	5	4	6	2	4
Singletons	0	5	1	1	5	2	4	6	1	4
Chao 1	2 (2/ 3)	19 (8/ 100)	7 (7/ 11)	4 (4/ 12)	21 (10/ 102)	10 (9/ 20)	18 (10/ 74)	26 (14/ 79)	2 (2/ 10)	12 (6/ 43)
Shannon	0.67	0.4	1.27	0.78	0.84	1.6	1.41	1.66	0.64	0.83
Evenness	0.9801	0.2136	0.5067	0.5438	0.2586	0.5513	0.4079	0.4773	0.9449	0.3814
Coverage*	100	95 %	99 %	99 %	95 %	98 %	96 %	94 %	67 %	96 %

S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching;

*Coverage based on Good´s formula $(1-(n/N)*100)$. Numbers in parenthesis indicate 95 % confidence intervals.

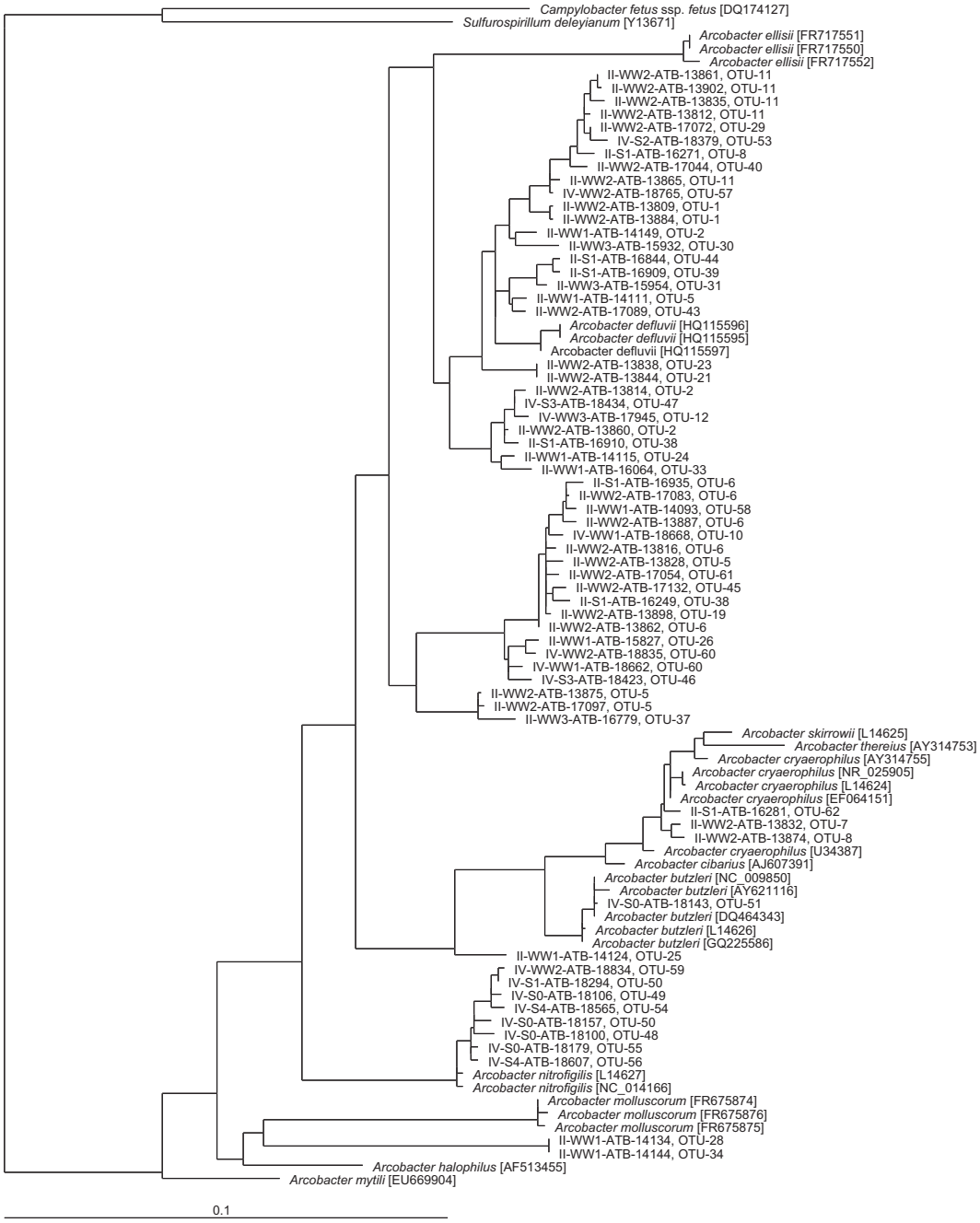

Figure 4.14.: Phylogenetic relationship of detected 16S rRNA gene sequences in comparison to reference sequences of *Arcobacter* species. Tree was constructed with the Maximum Parsimony algorithm (Kolaczkowski & Thornton, 2004). Accession numbers are indicated in brackets. Clones isolated in this study are designated with "ATB". Samples from 2007 are indicated with "II" and samples from 2009 with "IV". In case of minor differences inside of one OTU several sequences for this OTU were obtained.The bar represents 10% evolutionary distance.

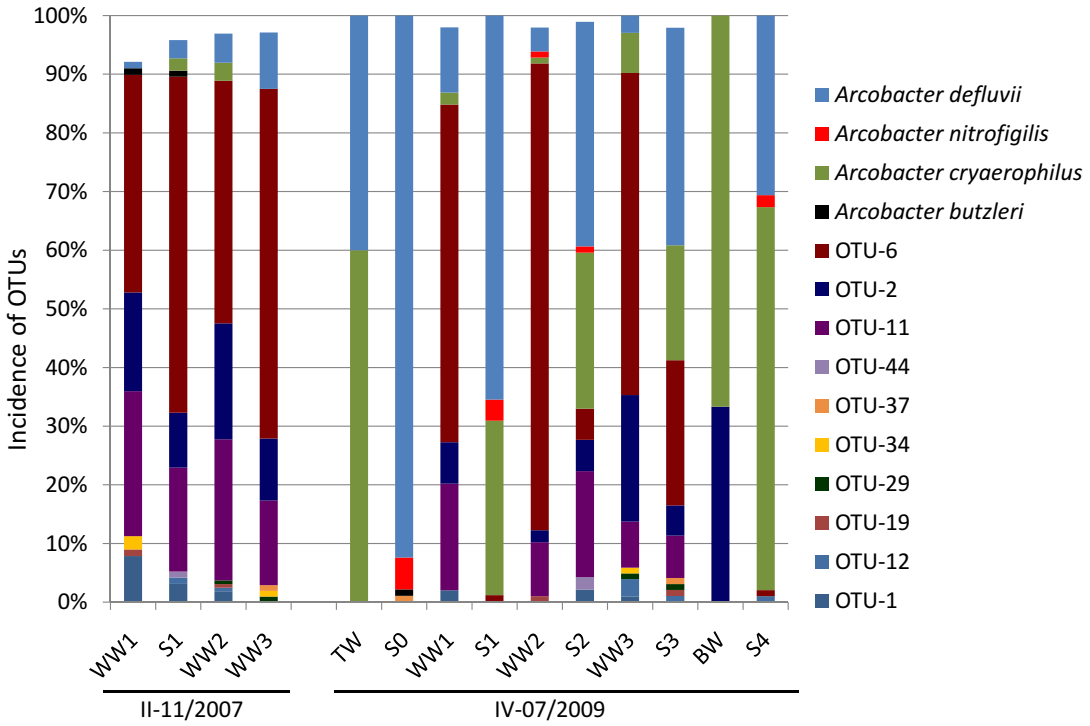

Figure 4.15.: Distribution of taxa as detected in the *Arcobacter*-specific clone libraries. The columns represent different sample sites in the plant. The colors indicate the incidence of the detected OTUs and species in the samples. S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching.

the most abundant being *Arcobacter cryaerophilus*. *Arcobacter butzleri* was detected in samples II-WW1, and II-S1 from 2007 and IV-S0 from 2009. Several OTUs were abundant in all samples and did not show enough similarity to be considered belonging to a known *Arcobacter* species (see Figure 4.15). The water samples had a distinctly different diversity compared to the spinach samples. They were dominated by OTU-6, OTU-2, and OTU-11. These OTUs were not detectable in IV-TW and IV-S0, but after two washing steps these OTUs could be detected on the spinach as well.

The samples of 2007 did show a similar diversity. Dominant OTUs were OTU-6, OTU-2 and OTU-11. Known *Arcobacter* species in these samples were *A. defluvii* and the pathogenic *A. butzleri* and *A. cryaerophilus*. The samples of 2009 were less divers. The sample of uncleaned spinach contained *A. butzleri*, *A nitrofigilis* and *A. defluvii*. The drinking water contained *A. defluvii* and *A. cryaerophilus*. After the first washing step *A. cryaerophilus* was also detected on the spinach. With each washing process the diversity on the spinach samples equaled more the

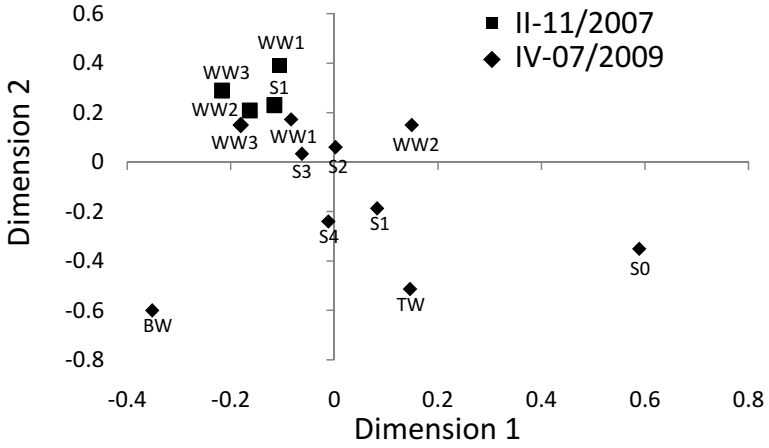

Figure 4.16.: Multidimensional scaling (MDS) of Chao-Jaccard indices from the *Arcobacter*-specific clone libraries of 2007 and 2009 visualizing the different diversities of the samples. S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching.

diversity of the wash water (see Figure 4.15).

The water from the blancheur and the blanched spinach showed a different diversity. Only a few OTUs were still detectable on the spinach, some of them were *A. cryaerophilus* and OTU-11. The wash water in the basins showed a distinctive diversity regardless of the time of sampling and the disinfection and cleaning procedures in the facility (see Figure 4.15). This suggests that *Arcobacter* species colonized the plant and are not removed by the cleaning procedures and survive during decommissioning of the plant in winter. The detected OTUs might be artefacts as the detection of DNA does not give any information about the viability of the cells.

The blanching step removes most of the OTUs typical in the wash basins, but *A. cryaerophilus* and *A. defluvii* were still detectable on the washed and blanched spinach. This shows that spinach can be contaminated during the washing process and that the contamination is not necessarily removed by the blanching process. This is supported by the Chao-Jaccard index. It compares diversity of each sample with the other samples (see Table C.1). Multidimensional Scaling (MDS) of the Chao-Jaccard indices shows the relationship of the samples in terms of their diversity (see Figure 4.16).

It can be seen that unclean spinach was quite different in comparison to the other samples, while the samples of 2007 clustered together. The samples of 2009 clustered together as well, but not as closely.

The analysis of the genetic diversity demonstrate that pathogenic and non-pathogenic *Ar-*

cobacter coexist in the spinach-washing plant. Please note, that the assay does not indicate whether the unknown *Arcobacter* species are pathogenic. So far, *Arcobacter defluvii* is not considered pathogenic and it can be assumed that the other unknown species belong to the group of environmental, non-pathogenic *Arcobacter* as well.

4.3.3. Isolation of *Arcobacter butzleri*

For an isolation of the undescribed *Arcobacter* sp. like *Arcobacter defluvii* several cultivation and isolation methods were used (see Paragraph 3.3), all samples of sampling IV-07/2009 were directly diluted and plated onto Blood Agar plates, *Arcobacter* broth plates with 5 % horse blood and SS1 or CAT supplement and on marine agar. Additionally, the filter method was used before spreading on Blood Agar plates and marine agar. *Arcobacter* colonies were only grown after an enrichment in ASM and plating on ASM-plates as described by Houf et al. (2001). Colonies were picked and identified by 16S rRNA gene sequencing and proved to be *A. butzleri* in all cases. As this method was originally developed to detect *A. butzleri*, *A.cryaerophilus* and *A. skirrowii*, this result was not surprising.

Although an isolation of unknown *Arcobacter* species was unsuccessful, this study shows that pathogenic and viable *A. butzleri* strains exist in the spinach-washing plant. The results obtained with the clone libraries show that *A. butzleri* seems to be present only in smaller numbers compared to the other OTUs and that it is not present in the process line all the time.

Therefore, it would be useful to monitor the microbial status of the post harvest process quickly and reliably. *Arcobacter* is slow growing and fastidious and, as can be seen in this study, many species are still unknown. Therefore, a detection by selective media is not fast enough considering that the quality of quickly degrading food is concerned and detection is impossible in cases where the growth conditions are not known. The existence of environmental *Arcobacter* species like *A. defluvii* in a sample may be an indicator for the possible occurrence of pathogenic species.

Several studies with the focus on *Arcobacter* determined the genetic diversity of isolated *Arcobacter* strains from slaughterhouses, fecal samples, and meat (van Driessche & Houf, 2007, van Driessche et al., 2005, 2004, On et al., 2002, Houf et al., 2002, Ho et al., 2008, Son et al., 2007). All these studies isolated *Arcobacter* strains after enrichment and analyzed the isolates with a multiplex-PCR (Houf et al., 2000) or by restriction fragment length polymorphism (RFLP) (On et al., 2003, 2004). Subsequently they identified *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* which is not surprising as the isolation protocol was developed for these strains (Houf et al., 2001) and the PCR was developed to distinguish only these three strains. There is no study using a molecular approach without prior enrichment/isolation procedures. Therefore the possible outcome of these studies was limited considerably a priori.

4.4. Development of a multiplex-PCR detecting *Arcobacter* species

The qPCR-Assay developed for *Arcobacter* detection was shown to be specific for all *Arcobacter* species described at that time and may prove to be a useful tool for a fast and efficient way to evaluate if *Arcobacter* is present in a specific sample. But since only some species are considered pathogenic a better differentiation between the *Arcobacter* species is necessary to asses a health risk. It was shown by the 16S rRNA gene clone libraries and the growth on selective media that the genetic diversity of the *Arcobacter* species is great and pathogenic species exist alongside non-pathogenic species. A multiplex-PCR was developed in order to distinguish between the *Arcobacter* species during a single PCR run (see Table C.3).

To check for cross-contamination between primer pairs, a primer mix was created containing all primers. Each gDNA was added to the PCR separately. The PCR products were visualized on a metaphorgel for a higher resolution (see Figure 4.17). It can be seen that for all *Arcobacter* spp. a product was obtained and that no undesired fragments were produced. In one sample all primer pairs and all gDNA was added. In this sample not all products were amplified, most likely because of competition between the primer pairs for the targets as some target genes share a primer.

In a next step the optimal annealing temperature was determined. At a temperature of 55 °C for all seven *Arcobacter* spp. an amplicon was produced. The limit of detection was determined to be at 50 pg of each *Arcobacter* gDNA (see Figure 4.17). This multiplex-PCR was used to screen the wash water samples, samples spiked with *A. butzleri* and *A. cryaerophilus* and enrichment cultures for *Arcobacter* (see Table 4.6). In the samples of V-10/2009 no *Arcobacter* species was detected, the *A. butzleri* cells the samples had been spiked with were only detected in two samples. These results suggests that the PCR was inhibited, e.g by humic acids and explains the inconsistent results with the qualitative PCR-Assay (see Section 4.2.3).

The samples of sample batches VI, VIa and VII were spiked with *A. cryaerophilus* cells, which were detected successfully in all spiked samples. No *Arcobacter* was detected in most direct PCRs, where the samples were not enriched prior to the PCR. Only in VII-WW2 *A. cryaerophilus* was detected. The occurence of *A. cryaerophilus* was also detected in the samples III-05/2008 in the clone library (see Section 4.3). After enrichment with Arcobacter broth and 5 % horse blood and CAT, *A. butzleri* was detected in all samples of sampling VII- and in VI-WW2 and on the cleaned spinach VI-S4. *A. skirrowii* was detected in enrichment cultures with *Arcobacter* broth + SS1 in most samples of VI and VII, especially in the water samples and on the cleaned spinach (VI-S4), but also in the biofilm samples taken from the blancheur.

These results show that it is possible to differentiate up to seven *Arcobacter* species with a

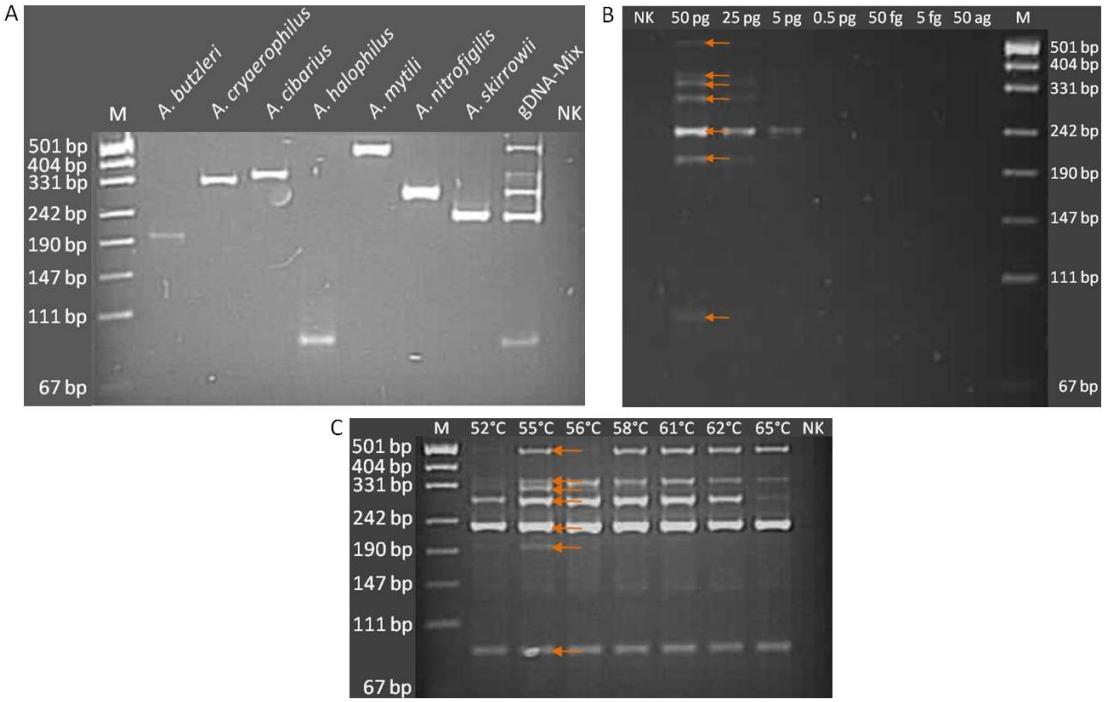

Figure 4.17.: Design of multiplex-PCR-Assay. (A) Test of specificity of multiplex-PCR. A mix containing all primer pairs were used with single gDNA templates. (B) Determination of the limit of detection (LOD) with amounts of gDNA. Amount is given per single gDNA. (C) Determination of optimal annealing temperature. M = marker; NK = negative control; arrows indicate positive amplifications.

Table 4.6.: Results of multiplex-PCR applied to samples from a spinach-processing plant which verified the existence of viable and pathogenic *Arcobacter butzleri* ("Butz"), *A. cryaerophilus* ("Cry"), *A. skirrowii* ("Skir") and non-pathogenic *A. halophilus* ("Halo").

V-10/2009	TW	S0	WW1	S1	WW2	S2	WW3	S3	BW	S4
original	-	-	-	-	-	-	-	-	-	-
spiked	-	-	-	-	-	-	Butz	-	-	Butz
VIa-04/2010										
original	n.a.	n.a.	-	n.a.	-	n.a.	n.a.	n.a.	n.a.	n.a.
spiked	n.a.	n.a.	Cry	n.a.	Cry	n.a.	n.a.	n.a.	n.a.	n.a.
VI-06/2010										
original	-	-	-	-	-	-	-	-	-	-
enriched/ blood	-	-	-	-	Butz	-	-	-	-	Butz
enriched/ SS1	-	-	Skir	-	Skir	-	Skir	Skir	-	Skir
spiked	Cry	Cry	Cry	Cry	Cry	Cry	Cry	Cry	Cry	Cry
VII-06/2010										
original	n.a.	n.a.	-	n.a.	Cry	n.a.	-	-	-	n.a.
enriched/ blood	n.a.	n.a.	Butz, Halo	n.a.	Butz, Halo	n.a.	Butz	Butz	Butz	n.a.
enriched/ SS1	n.a.	n.a.	Skir	n.a.	Skir	n.a.	Skir	Skir	-	n.a.
spiked	n.a.	n.a.	Cry	n.a.	Cry	n.a.	Cry	Cry	Cry	n.a.

S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching; n.a. = not analyzed

single PCR setup, but the sensitivity is not sufficient to detect *Arcobacter* directly in the samples. The cell counts without enrichment are too low. The 10^8 cells of *A. cryaerophilus* from the spiked samples were easily detected. It also supports the result that the majority of the *Arcobacter* cells detected in other experiments belong to other species than the seven that were targeted in this assay. Enrichment favors the species that were targeted in this assay, in particular *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*.

4.4.1. Extension of the multiplex-PCR

Recently, several new species were isolated and therefore it was tried to expand the multiplex-PCR to cover eleven species. Therefore, new primer pairs were designed and tested (see Table C.4). While nine species were easily detected, no PCR product was obtained for *A. butzleri* and *A. defluvii* (see Figure 4.18). With eleven, and nine amplicons, respectively, it was necessary to use capillary electrophoresis to visualize the PCR products as the differences in amplicon size were to small to be visualized by agarose gel electrophoresis. Adjusting the primer concentration and $MgCl_2$-concentration did not lead to improved results (see Figure C.4). Further experiments optimizing the annealing temperature and the primer concentrations for single primer pairs should make it possible to amplify the last two species as well. All amplicons except the fragments from *A. butzleri* and *A. defluvii* can be seen (4.19). The peak of *A. marinus* is very high and should be adjusted by reducing the primer concentration. When all peaks nearly exhibit the

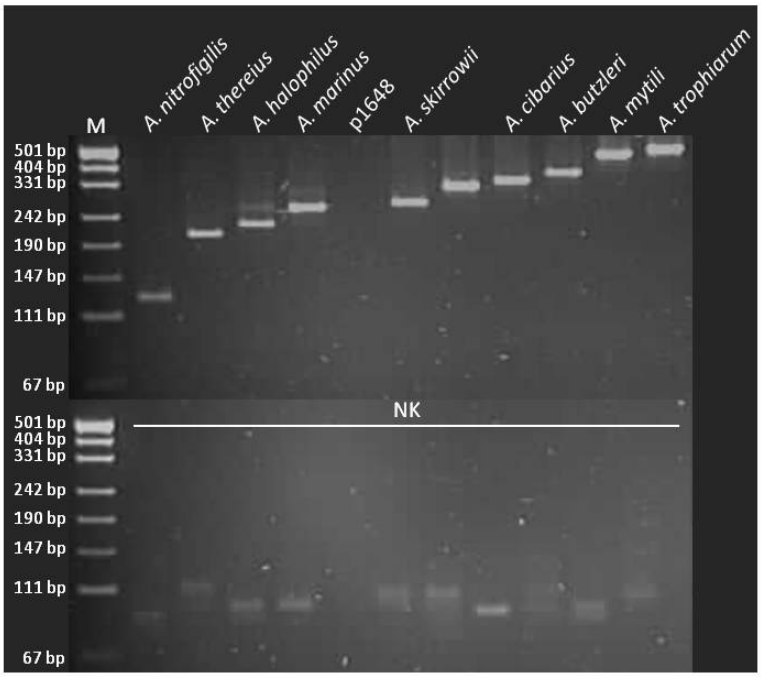

Figure 4.18.: Results of a PCR-Assay determining the specificity of multiplex primers. The PCR contained all primer pairs, but only one template gDNA. M = marker; NK = negative control.

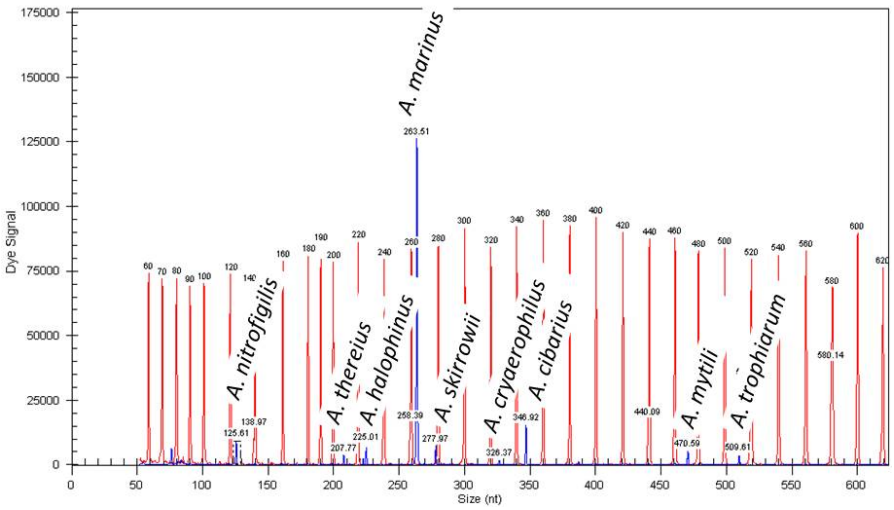

Figure 4.19.: Detection of products from multiplex-PCR by capillary electrophoresis. The blue line indicates the mixture of PCR fragments; the red line indicates the DNA standard.

same fluorescence, it may be possible to use a lesser dilution for the capillary electrophoresis and achieve a better visibility of the other peaks. Previously, Douidah et al. (2010) developed a multiplex-PCR which allows for the simultaneous detection of five *Arcobacter* species associated with animals. The amplicons were visualized with agarose gel electrophoresis. It was not possible to use the assay by Douidah et al. (2010) since the fragments were too long for a visualization by CE.

4.5. Detection of *Arcobacter* spp. by qPCR

A multiplex-PCR is a fast and reliable detection method, especially in combination with enrichment, but it does not allow for a quantification of bacteria. A qPCR-Assay for the detection and quantification of *Arcobacter* and *Pectobacterium* was developed and applied to the samples of the spinach-processing plant. Developing a multiplex qPCR detecting two or three species simultaneously is a very challenging task, therefore the qPCR was designed to be only genus-specific.

4.5.1. Development of an qPCR-Assay targeting the 16S rRNA gene of *Arcobacter*

The primer pair was based on the 16S rRNA gene, because the sequence of this gene is known for all *Arcobacter* species. The assay was designed to include all species of the *Arcobacter* genus. The assay consisted of the primer pair #436/#243 (see Figure 4.20).

Since the amplified fragment was 715 bp in length and the recommended amplicon length for a TaqMan assay is only 150 bp, the SYBR Green compound was used for fluorescent labeling of the amplified PCR products. The specificity of the assay was tested and a dissociation curve analysis was conducted in order to verify that only the intended amplicon was produced (see Figure 4.21). The standard curve showed an efficiency of only 61.6 %. Therefore another SYBR Green Mastermix (Fermentas) was tested. Efficiency was then increased to 98.9 %. The optimal primer concentration was established by a primer matrix where different concentrations of forward and reverse primers are used (see Figure 4.22). The optimal primer concentration is reached where the C_t values are lowest and the fluorescence values R_n are the highest. In this case the optimal concentration of forward primer #436 was 100 nM, while for the reverse primer #243 the best concentration is 80 nM.

Figure 4.20.: Alignment of 16S rRNA gene of *Arcobacter* sp. with primer binding sites (areas marked in grey). Accession numbers are indicated in brackets. Primer with their designation are shown in the first row.

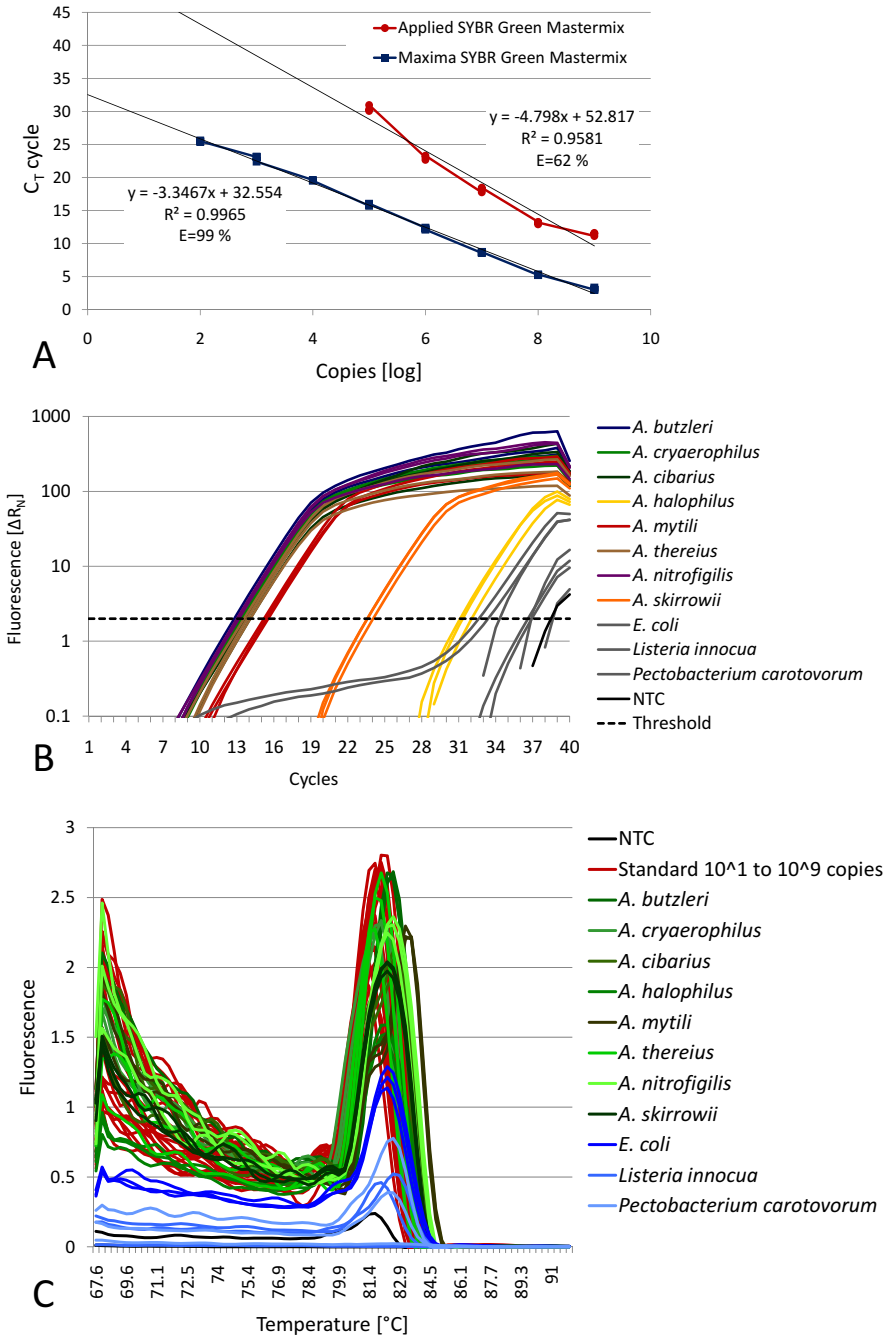

Figure 4.21.: Specificity and performance of 16S rRNA gene *Arcobacter*-specific qPCR-Assay. (A) Standard curves with two different mastermixes (red line = Applied Biosystems SybrGreen mastermix; blue line = Maxima SybrGreen mastermix; grey line = linear trendline. (B) Amplification plot with different gDNAs as templates showing the specificity of the qPCR-Assay. All tested *Arcobacter* strains are detected. *A. halophilus* is not detected by the assay. The graph shows the fluorescence over the cycle numbers. Background fluorescence has been cut off the graph. (C) Melting curve analysis of amplified fragments. NTC = no-template control.

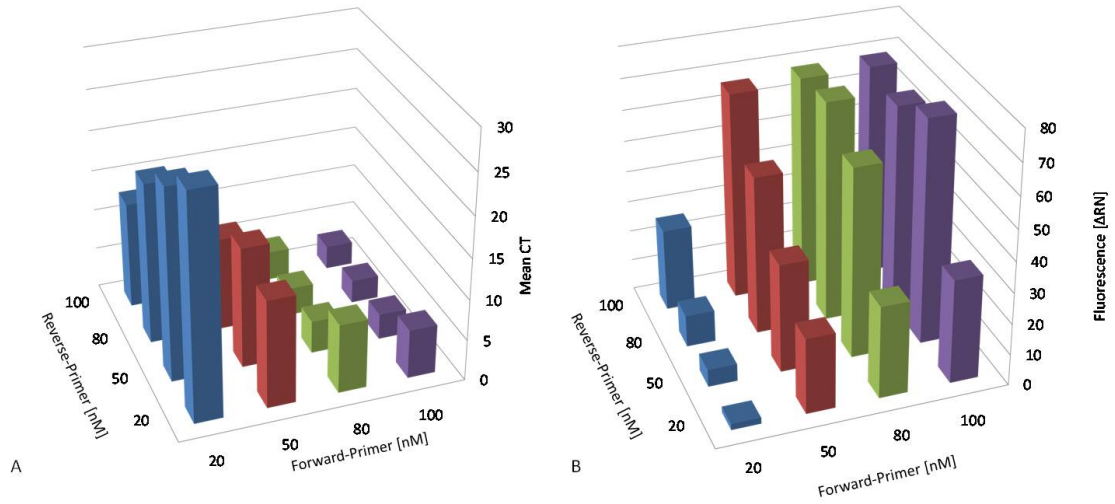

Figure 4.22.: Establishment of the optimal primer concentration for the 16S rRNA gene assay. The optimal primer concentration has low mean C_t-values and high R_N-values. (A) 3-D plot of primer concentrations against mean C_t-values. (B) 3-D plot of primer concentrations against fluorescence.

Spiking of samples

In order to evaluate the efficiency of DNA-Isolation and PCR-Amplification, each sample was spiked with 10^8 cells of an *A. butzleri* cell culture prior to DNA-Isolation. *Arcobacter butzleri* was chosen, because its genome was completely sequenced and the number of gene copies is known. The latter allows to calculate the cell number from detected gene copy numbers after the qPCR run. Another PCR-Assay was used for the detection of *A. butzleri* (Brightwell et al., 2007). It is based on the *rpo* gene, which codes for the RNA polymerase subunit B and is a single copy gene. An *A. butzleri* cell culture was tested with the assay in order to verify if there is a difference in the detection of a cell culture of *A. butzleri* and cells of *A. butzleri* added to gDNA (see Figure 4.23). No difference of the amplification was observed.

Spiked and original samples from the spinach-processing plant were tested with this assay. Results show that an amplification in the unspiked samples occurred (see Figure 4.24). Two possible reasons are conceivable: either *A. butzleri* occurs in the unspiked samples as well or the assay is not specific. Therefore the specificity of the assay designed for *A. butzleri* was tested with the SYBR Green chemistry to check for unspecific PCR products. Additionally, an assay with a primer pair specific for *A. cryaerophilus* was tested (Brightwell et al., 2007) and showed a better specificity (see Figure 4.25).

Therefore, samples from VIa-04/2010 onward were all spiked with 10^9 *A. cryaerophilus* cells. The *A. cryaerophilus* primer pair targets the 23S rRNA gene. To date the genome of

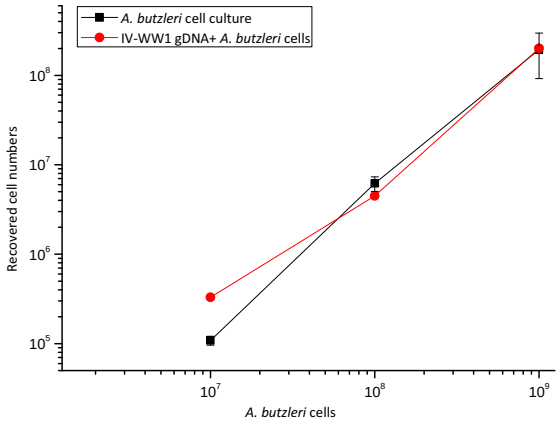

Figure 4.23.: Recovery rate of *A. butzleri* cells from a cell culture and from a spiked gDNA of the IV-WW1 sample. Recovered *A. butzler*i cells from genomic DNA of sample IV-WW1 spiked with cells of *A. butzleri* are indicated as red. Recovered *A. butzleri* cells are indicated in black. X-axis shows number of *A. butzleri* cells used and y-axis shows amount of recovered *A. butzleri* cells, error bars indicated standard deviation of triplicates.

A. cryaerophilus has not been sequenced and the number of gene copies of the 23S rRNA gene is unknown. In the following calculations of cell quantities a gene copy number of 4.5 was assumed, which is the mean copy numbers of the 23S rRNA gene in the genomes of *A. butzleri* and *A. nitrofigilis*. The spiking experiments of all samples showed that recovery rates of the cells were good. In general a loss of $10^1 \ldots 10^2$ cells was observed, even with the samples of a cell culture. This can be attributed to losses during DNA-Isolation, higher losses can be attributed to the inhibition of the PCR by substances like humic acids in the samples, as can be observed in the samples V-TW, V-BW, V-S3.

4.5.2. Development of an qPCR-Assay targeting the 23S rRNA gene of *Arcobacter*

The SYBR Green compound is sensitive to unspecific annealing of primer and primer dimer constructions. The TaqMan compound is more robust in this regard and allows for a better specificity. Unfortunately, it was not possible to find three suitable sites on the 16S rRNA gene that would be specific for the genus *Arcobacter*, but at the same time excluding all other bacteria, especially members of the Epsilonproteobacteria. Three sites are necessary for the forward and reverse primer and the TaqMan probe. Additionally, amplicons for a TaqMan assay should not exceed 150 bp in length. A requirement which could not be met based on the 16S rRNA gene. Therefore, several primers were designed for a new assay based on the 23S rRNA gene using the TaqMan compound (see Figure 4.26).

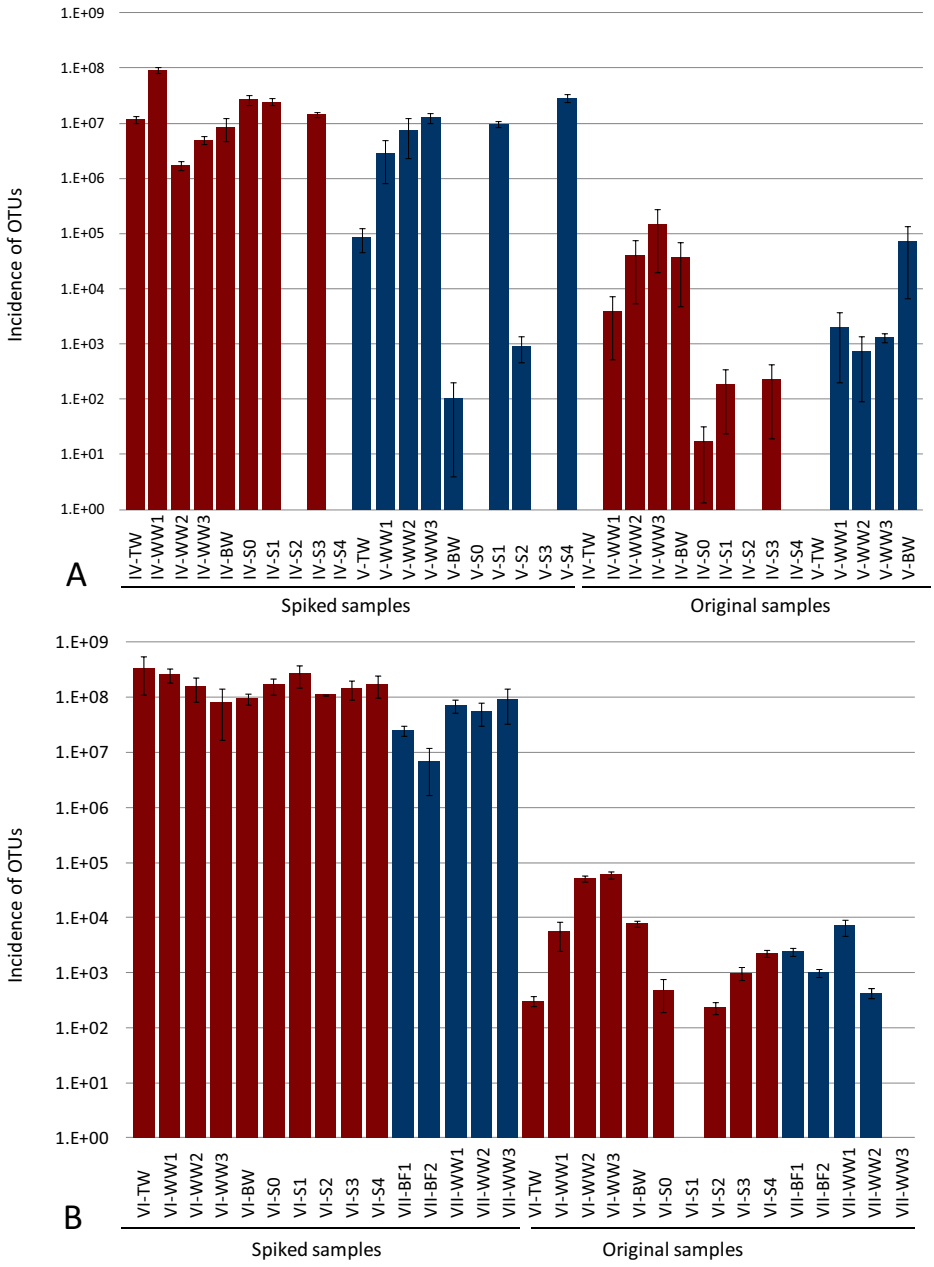

Figure 4.24.: Recovered cells from all spiked and unspiked samples with assays specific of *A. butzleri* and *A. cryaerophilus*. Error bars represent standard deviation of triplicates. (A) Samples from IV-07/2009 (red columns) and V-10/2009 (blue columns) spiked with *A. butzleri*. (B) Samples from VI-06/2010 (red columns) and VII-06/2010 (blue columns) spiked with *A. cryaerophilus*. S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching; BF1 = slimy biofilm from inside the blancheur; BF2 = hard biofilm from inside the blancheur.

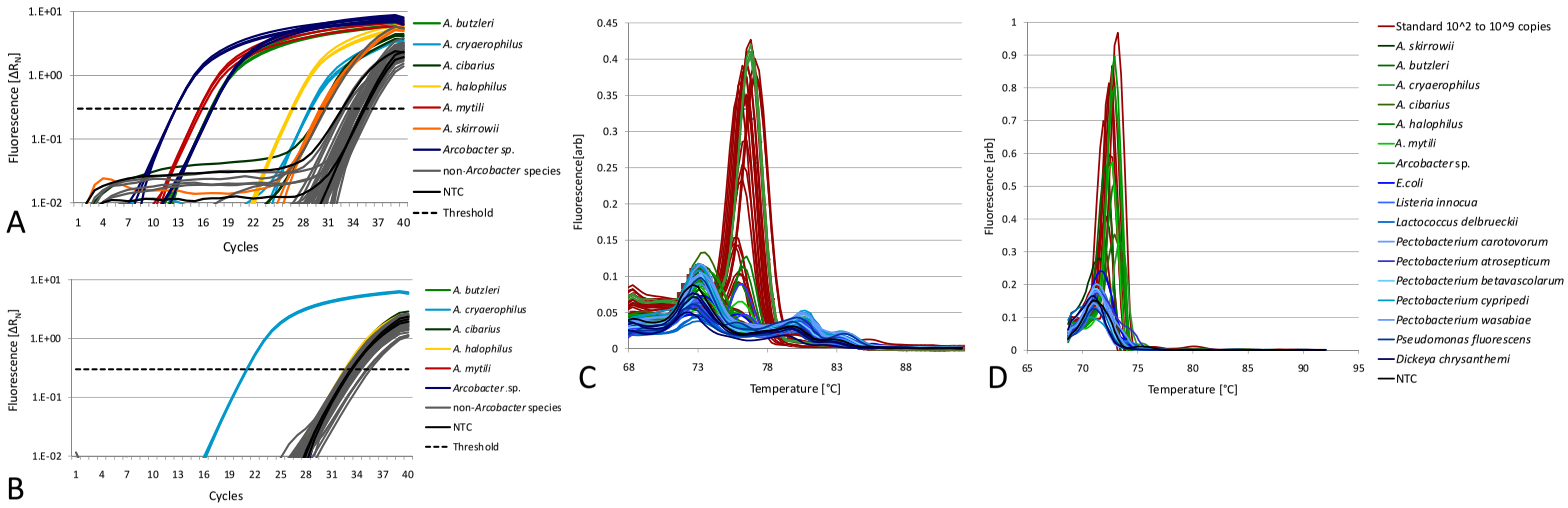

Figure 4.25.: Analysis of the specificity of an Arcobacter-specific qPCR-Assay (Brightwell et al., 2007). (A) Amplification plot with a primer pair specific for *A. butzleri* and gDNA of different *Arcobacter* species. Successful amplification occurred with *A. butzleri*, *A. mytili* and plasmid DNA of *Arcobacter* sp., a strain which was identified in this study. (B) Amplification plot with a primer pair specific for *A. cryaerophilus* and gDNA of different *Arcobacter* species. Successful amplification occurred with gDNA of *A. cryaerophilus*. Graphs (A) and (B) show the fluorescence over the cycle numbers. Background fluorescence has been cut off the graph. From the intersection point of threshold and amplification curves the C_t-values are obtained. (C) Melting curve analysis of fragments of *A. cryaerophilus* shows specific amplification of *A. cryaerophilus*, but no primer dimers or other unspecific products. (D) Melting curve analysis of the fragments of *A. butzleri* assay shows unspecific amplification of *A. mytili* and *Arcobacter* sp., but no primer dimers or other unspecific products.

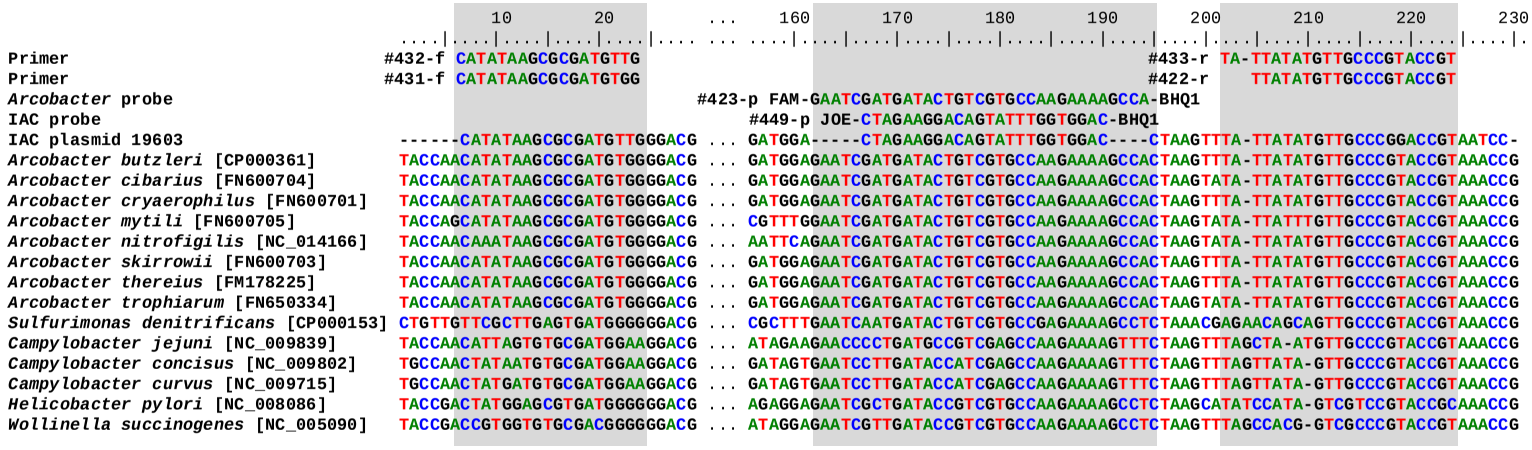

Figure 4.26.: Alignment of 23S rRNA gene sequences showing primer and probe binding sites (areas marked in grey). Accession numbers are shown in brackets.

However, some 23S rRNA gene sequences are not available yet, for example sequences of *A. defluvii*. Primer #431 and #432 are nearly the same, #432 contains a mismatch at the penultimate base to increase specificity. The amplified fragment has a length of 208 bp, which is longer than the recommended amplicon length for a TaqMan assay of 150 bp. Therefore, the probe was designed to be as close to the reverse primer as possible to make sure that the polymerase immediately moves along the probe and digests it when it is attached to the primer. The probe has the same orientation as the reverse primer and was labeled with 6-carboxyfluorescein (FAM) at the 5´-end and with a black hole quencher (BHQ1) at the 3´-end. All primer combinations were tested for their specificity (see Figure 4.27).

In order to check for undesired primer dimers and unspecific fragments the SYBR Green compound was used and a melting curve analysis was conducted. Based on the specificity and the standard curves the primer combination #432/#433 was selected and tested with the TaqMan compound and the *Arcobacter* specific probe #423 (see Figure 4.28). The resulting standard curve of this PCR run did not show the typical shape necessary for adequate quantification. Only seven of nine dilutions of the standard were detected. As the quality of the standard curve with the SYBR Green assay was satisfactory, it was assumed that the used mastermix was disadvantageous. Another mastermix (Fermentas) was used, which resulted in a significantly improved performance (see Figure 4.28).

IAC construction

For the control of the PCR efficiency an IAC was constructed. The performance of the IAC fragment in a qPCR was tested (see Figure 4.29). In all qPCRs where the 23S rRNA gene assay was used, 10^8 copies of IAC were added and primer in a concentration of 900 nM. In average $9.12 \cdot 10^6$ copies were recovered, regardless if a sample was tested or the IAC control. This indicates that no inhibition by substances in the samples occurred. The reduction in copy numbers could be caused by competition of the primer pair for the target gene. This could lead to a reduced efficiency. The detected quantities are shown in Table 4.30.

4.5.3. Detection of *Arcobacter* by qPCR

Arcobacter was detected in none of the samples with the 23S rRNA gene assay (data not shown). With the 16S rRNA gene assay *Arcobacter* was detected in the wash water samples of the samplings VIa-04/2010, VI-06/2010 and VII-06/2010. The difference can be explained with the different range of the assays. The 23S rRNA gene assay does not detect several species of *Arcobacter* like *A. defluvii* while the 16S rRNA gene assay has a broader range. This suggests that *Arcobacter* species other than the ones detected with the 23S rRNA gene assay exist in the

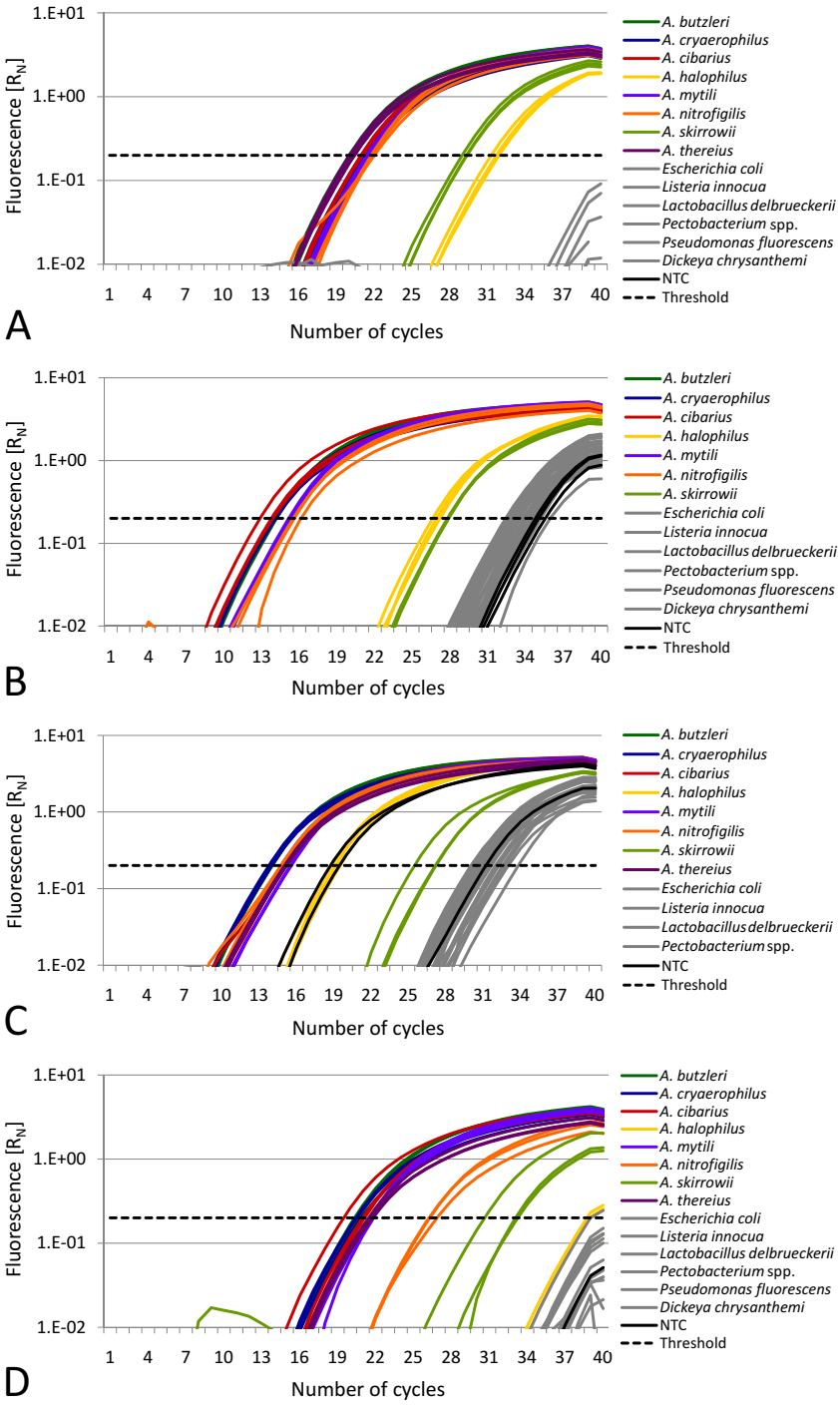

Figure 4.27.: Comparison of different primer combinations for an *Arcobacter*-specific 23S rRNA gene assay. Background fluorescence has been cut off the graph. (A) Amplification plot for primer pairs #432/#433, which were used for the 23S rRNA gene assay. (B) Amplification plot for primer pairs #431/422. (C) Amplification plot for primer pairs #431/#433. (D) Amplification plot for primer pairs #432/422.

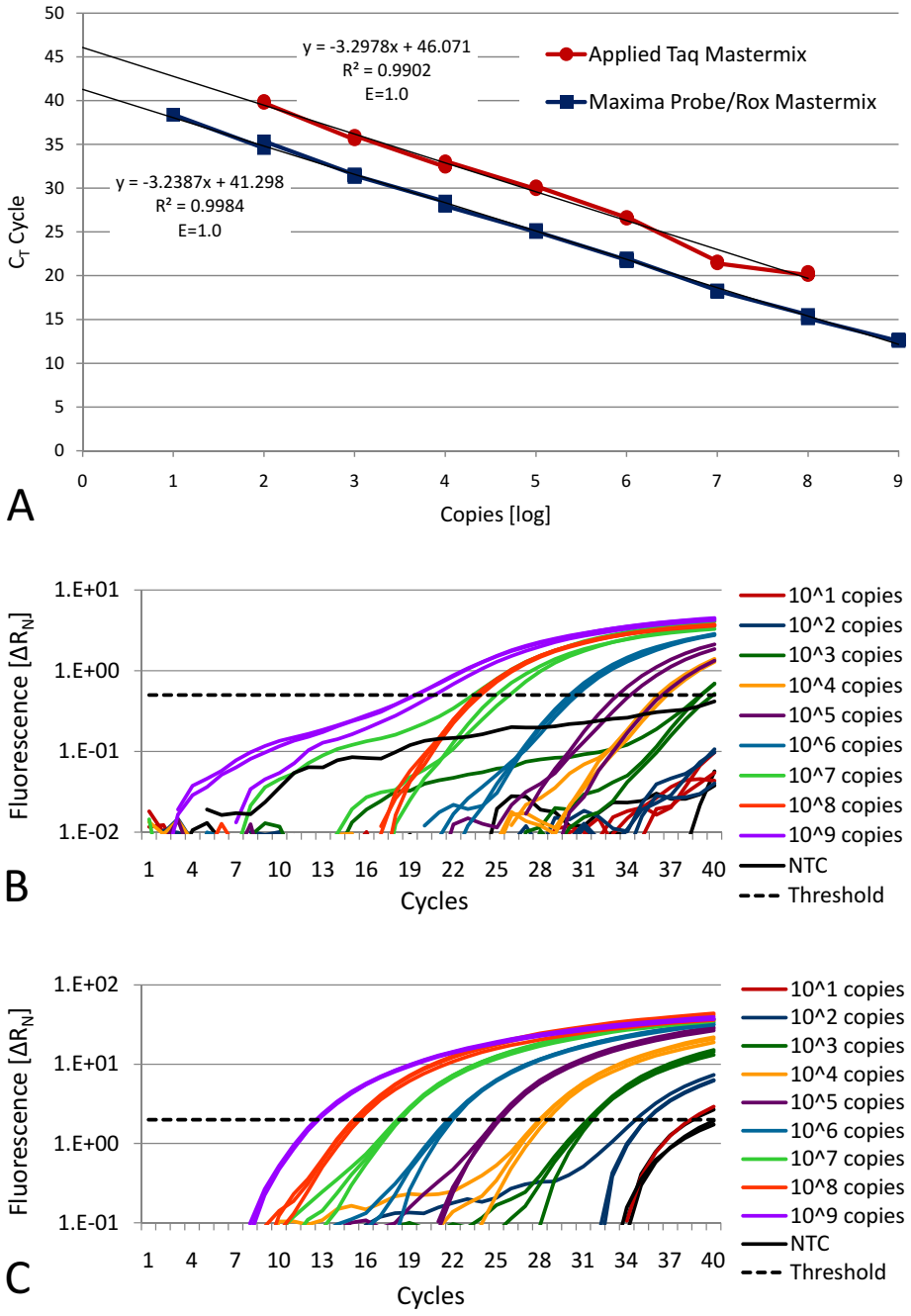

Figure 4.28.: Test of the 23S qPCR-Assay on standard dilutions with two different mastermixes showing the better performance of the Maxima Probe/Rox Mastermix. (A) Comparisons of standard curves for qPCR with Applied Biosystems Mastermix and Maxima Probe/Rox qPCR Mastermix. (B) Amplification plot of standard dilutions with Applied Biosystems Mastermix. Background fluorescence has been cut off the graph. (C) Amplification plot of standard dilutions with Maxima Probe/Rox Mastermix. Background fluorescence has been cut off the graph.

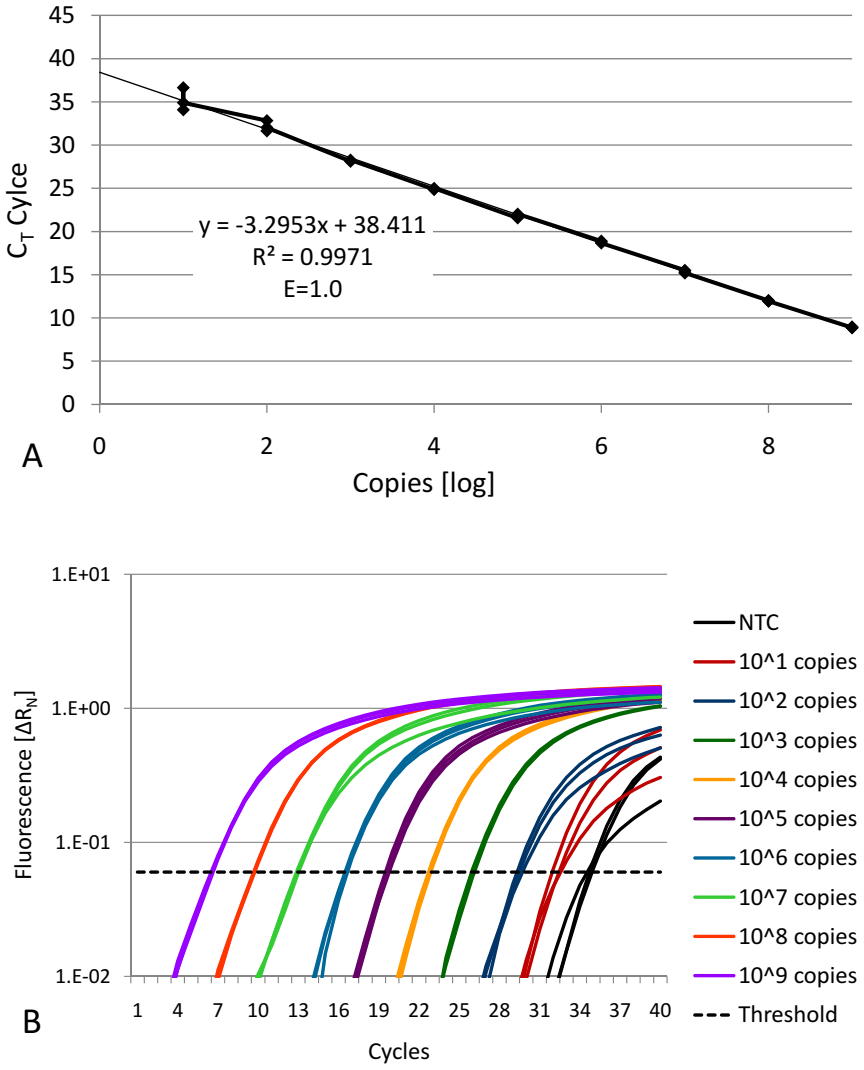

Figure 4.29.: Performance of IAC standard. (A) Standard curve of IAC standard plasmid. (B) Amplification plot of dilutions of standard plasmids. E = efficiency of PCR run, R^2 = coefficient of determination, Background fluorescence has been cut off the graph.

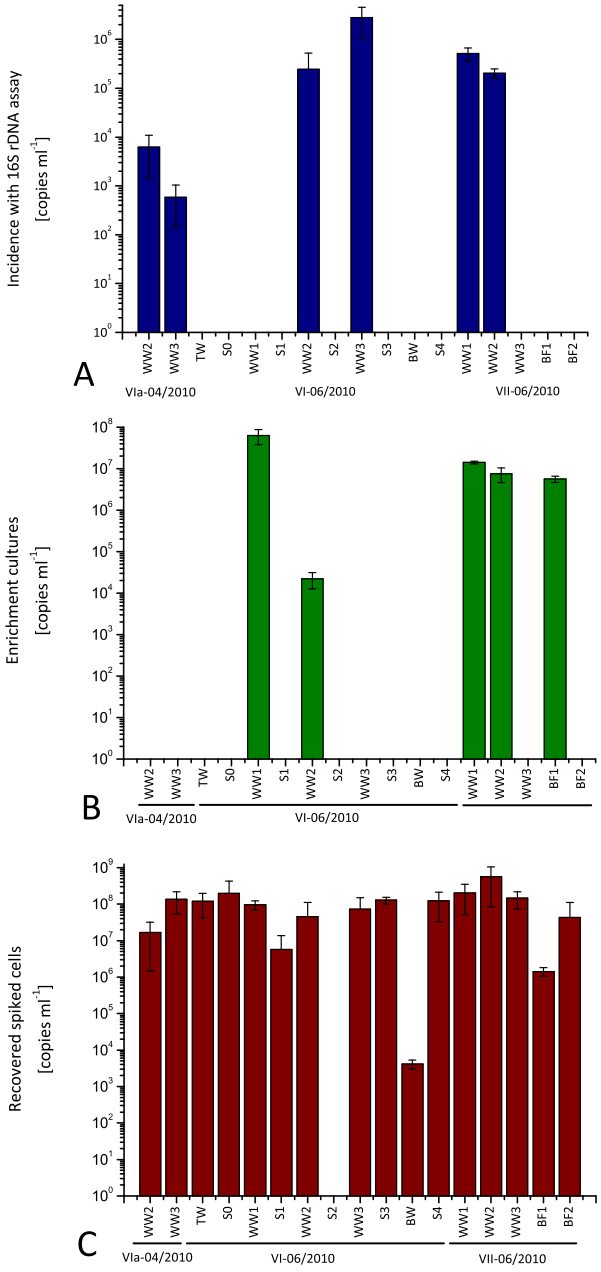

Figure 4.30.: Incidence of *Arcobacter* sp. in samples of spinach-processing plant by qPCR. (A) Detection of *Arcobacter* sp. with 16S rRNA gene assay. (B) Detection of *Arcobacter* sp. in enrichment cultures of samples of spinach-processing plant with 23S rRNA gene assay. (C) Recovered *A. cryaerophilus* cells spiked into samples detected by qPCR. S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching; BF1 = slimy biofilm from inside the blancheur; BF2 = hard biofilm from inside the blancheur; error bars indicate standard deviation of triplicates.

samples in numbers high enough to be detected by direct PCR. Some samples were positive with the 23S rRNA gene assay after enrichment. This shows that species covered by the 23S rRNA gene assay exist in small, undetectable numbers in the sample, which grow during the enrichment. Therefore these cells survive in a viable and cultivable state in the samples. Several samples showed inhibition of the PCR, e.g. samples VI-S2, VI-S1, VI-BW and VII-BF1. All inhibited samples did not show any amplification.

These results support the conclusions drawn from the experiment with the qualitative *Arcobacter* assay that *Arcobacter* survives in the water of the washing plant over a long period of time and is dispersed when production is continued. It is also shown that *A. defluvii* and the unknown *Arcobacter* species are suppressed by the enrichment procedure as for example VI-WW3 contains *Arcobacter*, but no *Arcobacter* species are detectable after enrichment. The enrichment is primarily optimized for the known pathogenic species *A. butzleri*, *A. cryaerophilus* and *A. cibarius*. Both assays are useful in detecting *Arcobacter*. The 23S rRNA gene assay detects the most important species including the pathogenic species and is less prone to false amplification because of the TaqMan mechanism. Some species are not detected with this assay, since a 23S rRNA gene sequence was not available for all species during primer design. The 16S rRNA gene assay detects all known and some unknown *Arcobacter* species, but since it is used with the SYBR Green compound unspecific amplification of some other species is more likely.

Recently, another genus-specific qPCR-Assay was published (González et al., 2010). It uses the SYBR Green compound and was tested on *Arcobacter* species. The primer pair (Bastyns et al., 1995) targets the 23S rRNA gene. It was tested with five *Arcobacter* species and shows a high sensitivity, even when used directly without enrichment step (González et al., 2010).

4.6. Detection of *Pectobacterium* spp. by qPCR

An assay was constructed for the detection of *Pectobacterium* which targets the *mdh*-gene. This gene codes for the malat-dehydrogenase enzyme. It is a housekeeping gene and exists only once in the genome. The assay was first developed for the SYBR Green chemistry. Later, a TaqMan probe was added (see Figure C.3). The specificity was tested with several strains of *Pectobacterium* species and other Gammaproteobacteria (see Figure C.2).

The standard curve showed an optimal efficiency of 1.0. The limit of detection (LOD) and limit of quantification (LOQ) were calculated to be at 33.0 and 32.8 cycles, respectively. Dissociation curve analysis showed no amplification of undesired fragments (see Figure 4.31). The optimal primer concentration was determined to be 100 nM.

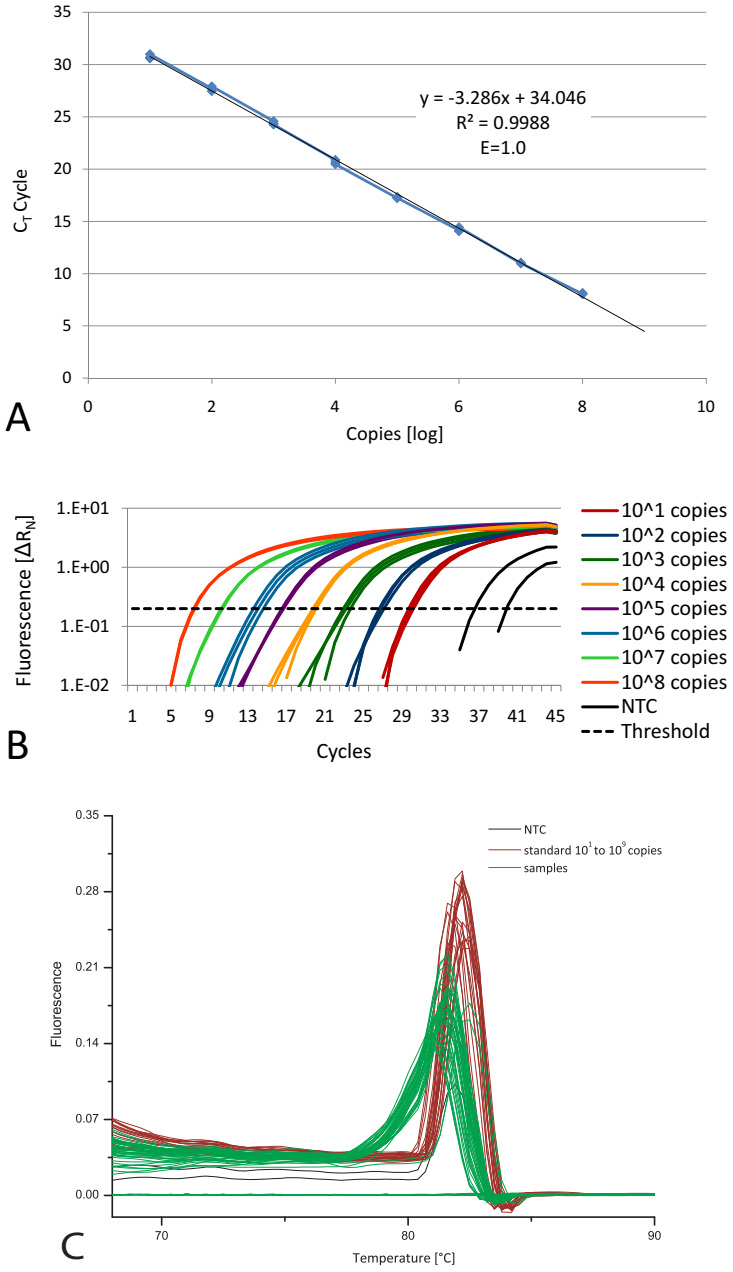

Figure 4.31.: Performance of qPCR-Assay specific for *Pectobacterium* spp. (A) Standard curve (B) Amplification plot of standard plasmid. Background fluorescence has been cut off the graph. (C) Melting curve analysis of amplified fragments. NTC = no-template control, E = efficiency of PCR run, R^2 = coefficient of determination.

Detection of *Pectobacterium* samples from spinach-processing plant The assay was used to detect *Pectobacterium* spp. in samples from samplings IV-07/2009 and V-10/2009. In both cases the entire process line was sampled. In the V-TW, the V-S0 and V-S2 no *Pectobacterium* spp. were detectable. All other samples were positive including the sample V-S4 of clean and blanched spinach. Compared with the results of the spiking experiments and of the qualitative PCR-Assay it has to be assumed that some of these results are false. The spiking experiments suggest that PCRs with samples V-TW, V-S0, V-S2, V-S3 and V-BW were inhibited and cannot be analyzed.

From samples V-S0 and V-S3 no spiked cells were recovered and from V-TW and V-S2 only a reduced number could be recovered. This suggests that the gDNA of V-10/2009 contained inhibitors. The cell numbers of the sampling V-10/2009 are very low, probably because of inhibitors in the gDNA, but in sampling IV-07/20009 cell numbers were considerably higher. The highest amount was detected in sample IV-WW3 (see Figure 4.32).The inhibition of the PCR in sampling V-10/2009 could also explain the inconsistent result obtained with the qualitative PCR (see Section 4.2.6).

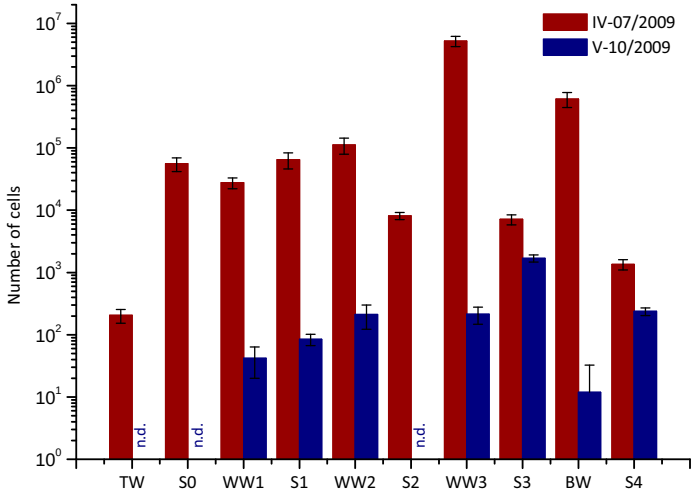

Figure 4.32.: Quantity of *Pectobacterium* in IV-07/2009 and V-10/2009 established by qPCR. The horizontal axis indicates different sample sites in the plant: S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching. n. d. = not detected; error bars indicate standard deviation of triplicates.

4.7. Establishment of the diversity by culture-dependent MALDI-TOF MS of a spinach-processing line

4.7.1. Description of method setup

The identification of bacterial colonies is a novel application of MALDI-TOF MS based on the entire protein spectrum of the colonies. Bacteria can be identified with high accuracy by comparison with spectra in a reference database. For this approach samples of the spinach washing plant were plated on standard I agar. Grown colonies were transferred onto fresh plates to produce a sufficient amount of cell material for MALDI-TOF MS and sequencing if necessary.

451 colonies were evaluated with the MALDI-TOF MS. The resulting spectra were analyzed with the SARAMIS software and compared to the SARAMIS reference database. Most colonies were not identified since the focus of the database is on clinically relevant bacteria. A cluster analysis was conducted and colonies representing an operational taxonomic unit were used in a PCR to amplify the 16S rRNA gene gene for sequencing analysis.

4.7.2. Establishment of diversity

The comparison with the SARAMIS database resulted in matches for 47 % of the analyzed colonies, but in most cases the match showed only $40-60$ % of similarity of the spectra. The similarity should be over 90 % for a reliable positive match. In order to confirm these results and to identify all other colonies, all spectra were used in a cluster analysis. Colonies with a similarity of more than 50 % were considered to be one OTU (Munoz et al., 2011). Table 4.7 gives an overview over the viable count of the samples, the number of results obtained by MALDI-TOF MS and the number of identified OTUs per sample.

A high percentage of colonies did not grow again on the second agar plate they were transferred to, leading to high losses in some samples. A rarefraction analysis showed that an undersampling occurred for all samples (see Figure 4.33) .

This is supported by the coverage parameter calculated with Good´s formula (Good, 1953). To evaluate changes in the diversity composition and distribution and whether the washing process leads to a reduction of the bacterial load, several diversity indices were calculated (4.7). As expected, we observed the highest microbial diversity in the beginning of the process in both sample types the spinach itself (S0 and S1) as well as the water of the wash basin 1 (WW1). Additionally our results showed that the diversity on the spinach increased after passing through the first wash basin whereby in the subsequent samples of the washing process the bacterial diversity is reduced, symbolized by a decreasing Shannon diversity index (see Table 4.7). The water sample from the blancher showed the lowest diversity which is not surprising, considering

Table 4.7.: Isolated colonies and MALDI-TOF MS results for each sample (V-10/2009) including diversity indices.

Sample	Viable c ells $[\text{cells} * \text{ml}^{-1}]$	No. of colonies analyzed by MALDI	detected OTUs per sample	Evenness	Coverage [%]*	Shannon	Chao 1
V-TW	0	-	-	-	-	-	-
V-S0	$7.0 * 10^4$	71	22	0.53	69	2.46	32 (24/61)
V-WW1	$1.3 * 10^5$	78	30	0.62	61	2.93	46 (34/82)
V-S1	$1.1 * 10^4$	77	26	0.54	66	2.64	42 (30/83)
V-WW2	$1.4 * 10^5$	47	17	0.51	63	2.16	53 (24/185)
V-S2	$5.8 * 10^3$	24	10	0.84	37	2.13	34 (13/177)
V-WW3	$6.4 * 10^4$	111	18	0.31	83	1.73	78 (27/393)
V-S3	$1.3 * 10^4$	63	10	0.62	47	1.83	42 (14/221)
V-BW	$1.0 * 10^2$	8	5	0.95	37	1.56	5(5/12)
V-S4	$2.0 * 10^5$	38	6	0.59	65	2.03	21 (14/55)

S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching;

*Coverage based on Good´s formula $(1 - (n/N) * 100)$

that the blanching process should eliminate most of the bacteria. After blanching the diversity of the blanched spinach rose slightly compared to the spinach in wash basin 3, but was still considerably lower than in the beginning of the wash process. The evenness has higher values for samples that are undersampled like sample V-BW and V-S3, while samples like V-WW3 have a low evenness value suggesting that species in the sample are not evenly dispersed, but instead some species are dominant. The evenness value seems to be sensitive to undersampling leading to a biased result suggesting that species in the samples are evenly distributed. The results for evenness should therefore be considered with caution. The values calculated for the Chao-I-Richness estimator are below the 95 % confidence intervals for all samples suggesting that many taxa were not identified due to undersampling. In order to compare the diversities of the samples the Chao-Jaccard index was calculated. The results are shown in Figure 4.34.

The graph is a two-dimensional representation of the Chao-Jaccard indices. No relationship can be seen between the samples. The wash water samples show a similar diversity, while the diversity of the spinach samples varies considerably. The blanched spinach differs from all other samples in its diversity which is not surprising, considering that the blanching process should eliminate most of the bacteria.

A principal component analysis (PCA) was conducted in order to determine if certain OTUs are dominant and influence the diversity of the samples. In principal component analysis the first

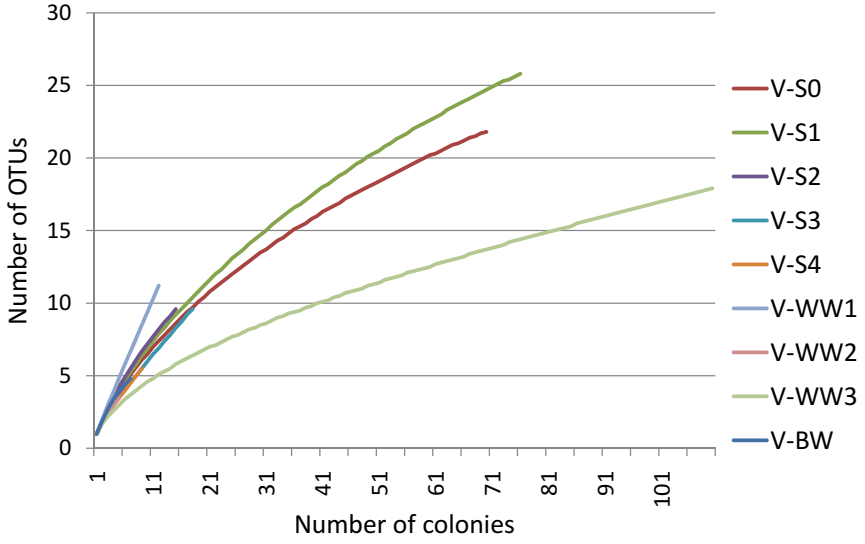

Figure 4.33.: Rarefraction analysis of OTUs detected by MALDI-TOF MS in samples of a spinach processing plant. Graph shows the number of identified OTUs over number of isolated colonies. S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching.

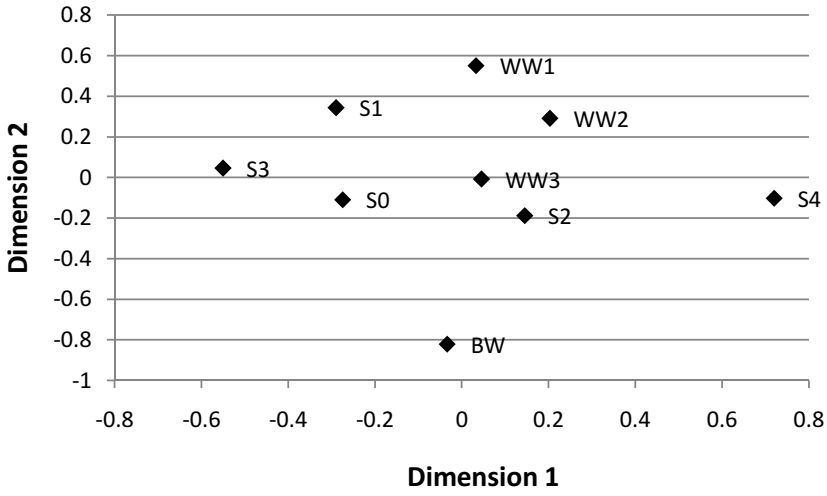

Figure 4.34.: Multidimensional scaling of Chao-Jaccard indices of samples from spinach processing plant analyzed with MALDI-TOF MS. S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching.

Table 4.8.: Summary of PCA analysis for OTUs of MALDI-TOF MS.

Importance of components:	Standard deviation	Proportion of Variance	% Proportion of Variance
PC1	4.5	0.24	24
PC2	4.1	0.20	20
PC3	3.7	0.16	16
PC4	3.3	0.13	13
PC5	2.8	0.09	9
PC6	2.8	0.09	9
PC7	2.1	0.05	5
PC8	1.6	0.03	3
PC9	$1.8 * 10^{-15}$	0	0

principal components should be responsible for 90 % of the variance. As can be seen in Table 4.8 the first two components of the data set do not represent 90 % of the variance, but no less than the first six.

This means that the diversity is not influenced by only one or two variables (in this case OTUs) and that no correlation is observable between the diversity of the samples. A graphical representation like a biplot is only possible for two components, therefore the biplot in (see Figure 4.35) only shows 44.72 % of the variance.

4.7.3. Phylogenetic analysis of isolated colonies

For every OTU the 16S rRNA gene sequence was obtained by PCR and sequenced. The sequences were compared to reference sequences from the SILVA and NCBI databases and phylogenetically analyzed (C.5). Table 4.9 shows the isolated species and their incidence in the samples of the spinach-processing plant. 50 % of the detected species belong to the class of Gammaproteobacteria. Firmicutes were represented with 22 %. Most species are unknown and in several cases more than one unknown species of a genus was isolated. Many isolated species belong to genera which also contain pathogenic or opportunistic pathogenic bacteria. In comparison to species identified by the 16S rRNA gene clone library in a sample of carrot wash water similar species were detected, at least regarding to the class of Gammaproteobacteria.

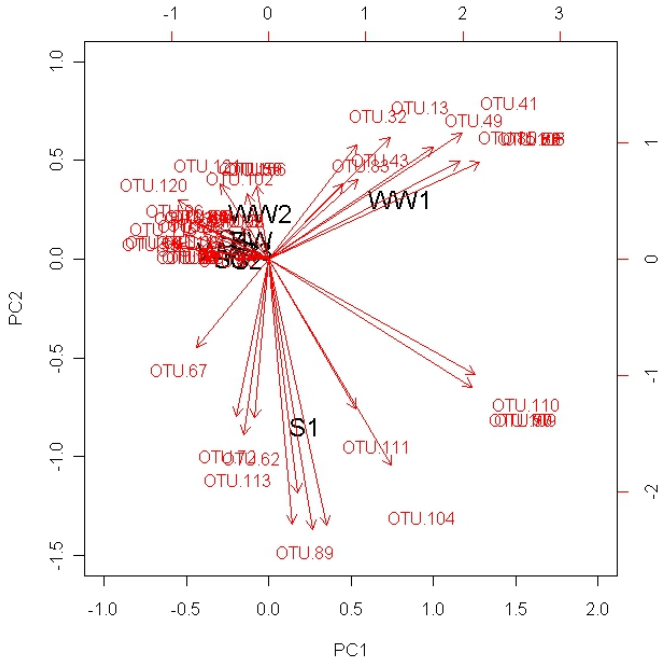

Figure 4.35.: Biplot of principal component 1 and principal component 2 representing 44 % of variance between samples. It shows the OTUs affecting the diversity in the samples. S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching.

A rarefraction analysis showed an undersampling, but not as pronounced as with the OTUs (see Figure 4.36). The values according to Good´s formula do not show an undersampling of the identified taxa as strongly as with the numbers for the OTUs (see Table 4.10).

The reason for the undersampling is that the MALDI-TOF MS is very specific in detecting differences and it turned out that sometimes several OTUs were defined for the same species.

The Shannon indices are lower than for the OTUs, but are still similar for the samples except the V-BW which shows a much lower diversity. The Chao-I-richness estimator shows an undersampling of most samples, since in most samples less taxa were identified than the lower confidence interval for the Chao-I estimator predicted. In case of the sample V-BW the sample size was nearly adequate, while the Chao-I estimator for the OTUs of that sample indicated a considerable undersampling. This demonstrates that the OTUs were not representative for taxonomical units, in this case, species. A similarity of 50 % was too high to distinguish between species. The evenness values of the samples are ranging from 0.33 for the sample V-S3 to 0.96 for the sample V-BW. This indicates that some samples are dominated by certain taxa, e.g. samples V-S3 and

Table 4.9.: Isolated species and their incidence in samples from a spinach-processing plant (cfu $*1^{-1}$ of sample).

Sample	S0	WW1	S1	WW2	S2	WW3	S3	BW	S4
Acinetobacter sp. ATB-1	0	0	0	$1*10^6$	0	0	0	0	0
Acinetobacter sp. ATB-2	0	$2*10^6$	0	$1*10^6$	0	0	0	0	0
Acinetobacter sp. ATB-3	0	$5*10^6$	0	$5*10^6$	0	0	$1*10^5$	0	$1*10^6$
Aerococcus sp.	0	$2*10^6$	0	0	0	0	0	0	0
Aeromonas sp. ATB-1	0	0	0	0	0	$1*10^5$	0	0	0
Aeromonas sp. ATB-2	$2*10^5$	$4*10^6$	$1*10^5$	$1*10^6$	0	$3.6*10^6$	0	0	$3*10^6$
Bacillus sp. ATB-1	0	$3*10^6$	0	0	0	0	0	0	0
Bacillus sp. ATB-2	0	0	0	0	0	0	0	$1*10^4$	0
Bacillus sp. ATB-3	$5*10^5$	$3*10^6$	$1*10^5$	$1.3*10^7$	$2*10^5$	$8*10^5$	$1*10^5$	$1*10^4$	0
Brachybacterium sp.	0	$3*10^6$	$1*10^5$	0	0	0	0	0	0
Comamonas sp. ATB-1	0	0	$1*10^5$	$1*10^6$	0	0	0	0	0
Comamonas sp. ATB-2	0	$1*10^6$	$1*10^5$	0	0	0	$1*10^5$	0	0
Comamonas sp. ATB-3	0	$2*10^6$	0	0	0	0	0	0	0
Enterobacteriaceae	$2*10^5$	0	0	0	0	$4*10^5$	$1*10^5$	0	0
Enterococcus sp.	0	$1*10^6$	0	0	0	0	0	0	0
Erwinia persicina	0	$1*10^6$	0	0	0	0	0	0	0
Lactococcus sp. ATB-1	0	0	$5*10^5$	0	0	0	0	0	0
Lactococcus sp.ATB-2	0	0	$1*10^5$	0	0	0	$1*10^5$	0	0
Microbacterium sp.	0	$3*10^6$	0	0	0	0	0	0	0
Pantoea agglomerans	0	$7*10^6$	0	0	0	0	0	0	0
Pantoea anthopila	0	$1*10^6$	0	0	0	0	0	0	0
Pantoea sp. ATB-1	$1.8*10^6$	$1*10^6$	$1.8*10^6$	$2*10^6$	$4*10^5$	$1*10^5$	$2.6*10^6$	0	0
Pantoea sp. ATB-2	$1*10^5$	0	$1*10^5$	0	0	$4*10^5$	0	0	0
Pantoea sp. ATB-3	0	$1*10^6$		0	0	0	0	0	0
Pantoea sp. ATB-4	0	$4*10^6$	$9*10^5$	0	$3*10^5$	$1*10^5$	$5*10^5$	0	0
Pantoea sp. ATB-5	0	$1*10^6$	0	0	0	0	0	0	0
Paraoerskovia sp.	0	$1*10^6$	0	0	0	0	0	0	0
Providencia heimbachae	$1*10^5$	0	0	0	0	0	0	0	0
Pseudomonas sp. ATB-1	0	0	$1*10^5$	$1*10^6$	0	$1*10^5$	0	0	0
Pseudomonas sp. ATB-2	0	0	0	$1*10^6$	0	0	0	0	0
Rahnella sp.	0	0	$1*10^5$	0	0	0	$1*10^5$	0	0
Raoultella sp.	$1*10^5$	$1*10^6$	$1*10^5$	0	0	$2*10^5$	0	0	0
Rothia sp.	0	0	$4*10^5$	0	0	0	$1*10^5$	0	0
Salmonella subterranea	0	0	0	0	0	0	0	0	$1*10^6$
Shewanella sp.	$2*10^5$	0	0	0	0	0	0	0	0
Staphylococcus sp. ATB-1	$1*10^5$	0	0	0	0	0	0	0	0
Staphylococcus sp. ATB-2	$1*10^5$	0	$2*10^5$	0	0	$1*10^5$	0	0	0
Staphylococcus sp. ATB-3	$1*10^5$	$5*10^6$	$7*10^5$	0	$4*10^5$	0	$1*10^5$	0	0
Streptococcus sp.	0	0	0	0	0	0	0	0	$1.2*10^7$

S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching.

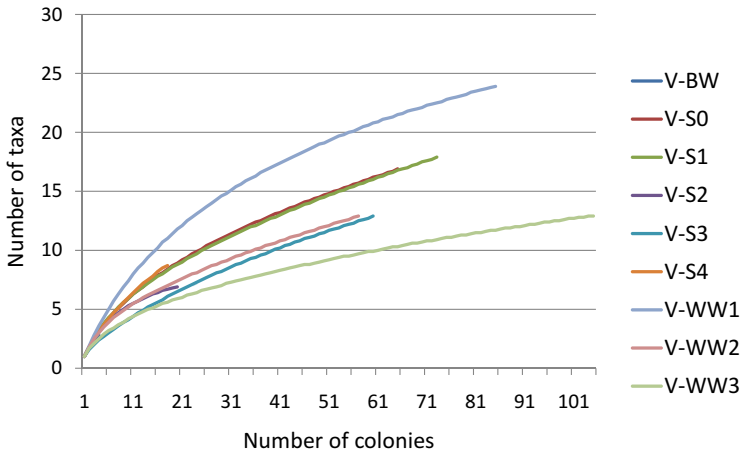

Figure 4.36.: Rarefraction analysis of phylogenetically assigned taxa identified by MALDI-TOF MS. S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching.

Table 4.10.: Diversity indices of taxa detected in samples from a spinach-processing plant.

Sample	Taxa	Evenness	Good [%]	Shannon	Chao 1
V-TW	-	-	-	-	-
V-S0	17	0.52	86	2.18	37 (20/120)
V-WW1	24	0.66	87	2.81	31 (24/58)
V-S1	18	0.51	49	2.22	43 (23/141)
V-WW2	13	0.53	83	1.85	24 (14/80)
V-S2	7	0.80	64	1.91	20 (9/101)
V-WW3	13	0.36	95	1.49	18 (12/52)
V-S3	13	0.33	85	1.52	54 (20/275)
V-BW	3	0.96	80	1.05	3 (3/7)
V-S4	9	0.71	87	1.66	11 (9/27)

S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching.

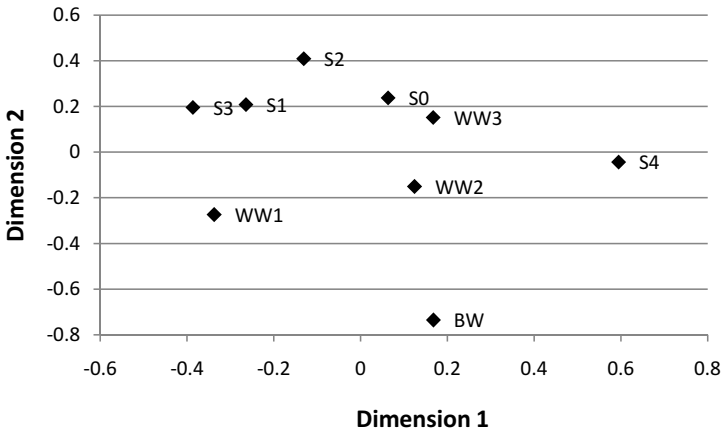

Figure 4.37.: Multidimensional scaling of Chao-Jaccard indices for taxa identified by MALDI-TOF MS. S0 = uncleaned spinach; TW = tap water; WW1, WW2, WW3 = water samples of wash basins 1, 2 and 3; S1, S2, S3 = samples of spinach from wash basins 1, 2 and 3; BW = water from blancheur; S4 = sample of spinach after blanching.

V-WW3, while in samples like V-BW, V-S5 and V-S2 species seem to be evenly distributed. The Chao-Jaccard index displayed by MDS shows some differences to the analysis with the OTUs (see Figure 4.37).

The V-WW1 sample shows a different diversity than the other wash water samples. The spinach samples are all similar to each other except for the V-S4 sample. The V-BW is different as well and does not cluster with any of the other samples.

This study shows that the use of MALDI-TOF MS as a tool for the establishment of diversity offers some advantages. It is a very fast and reliable method. The spectra are very specific and provide the necessary specificity to differentiate at least on the species, possibly even on the subspecies level. But the method depends on the selected media and has the same bias as other culture-dependent methods. It also depends on the existence of reliable databases with reference spectra. To date only a small amount of bacterial species has been analyzed and mostly those that are clinically relevant. Another advantage of the method in comparison to molecular methods is the possibility to assess the quantity of cells. Quantification of bacteria and the calculation of viable counts is very time-consuming when methods like qPCR and FISH are used. Additionally, it offers the possibility to use diversity estimators to evaluate the obtained results.

The methodical setup in this study had some disadvantages, because colonies were transferred onto a second plate to guarantee a sufficient amount of cell material for MALDI-TOF MS. This resulted in losses, because many colonies did not grow again on the second plate.

Cluster analysis proved useful, if a species could not be found in the database, but the exact

Table 4.11.: Comparison of diversity established by 16S rRNA gene clone library from carrot wash water and by MALDI-TOF MS from samples from a spinach-processing plant. The most dominant species in both samples were Gammaproteobacteria.

Samples from	Carrot wash water		Spinach wash water	
	Clones [%]	OTU	Colonies [%]	OTU
Actinobacteria	-	-	3	6
Bacteroidetes	14	17	<1	1
Firmicutes	14	15	25	29
Proteobacteria	71	62	56	42
Alphaproteobacteria	3	10	<1	1
Betaproteobacteria	10	22	2	3
Epsilonproteobacteria	11	2	<1	1
Gammaproteobacteria	47	28	54	37

line between species and subspecies is not well defined yet and grades of relationship cannot be ascertained from the cluster analysis algorithm incorporated in the Saramis software. However, it provides the possibility to reduce the sample size for sequencing. On the other hand even 16S rRNA gene gene analysis often does not lead to a conclusive identification of a colony in some genera since the gene is too conserved. This is the case for many Enterobacteriaceae (Case et al., 2007).

The analysis of the spinach processing line shows that the blanching step is effective in reducing the bacterial load. The washing step has no effect concerning the reduction of the bacterial load. In case of spinach, it is not the main purpose of the washing step to reduce the bacterial load, but to reduce the nitrate content of the spinach.

Comparing the results from the 16S rRNA gene clone library of the carrot-washing plant (see Section 3.2.1) with the results from the spinach-processing plant obtained by MALDI-TOF MS, it was shown that Proteobacteria, especially Gammaproteobacteria are dominant in both washing plants. Approximatly one third of the species identified by MALDI-TOF MS had also been detected in the 16S rRNA gene clone library. Firmicutes were identified in similar proportions in both habitats (see Table 4.11). With 14 % a considerable part of the 16S rRNA gene clone library was identified as Bacteroidetes, especially the class of Flavobacteria. In contrast only one species belonging to this class was identified with the MALDI-TOF MS. A reason for this difference may be that optimal growth conditions were not met in the preparation for MALDI-TOF MS. Plate-count agar which was used in this study is not the recommended medium for *Flavobacterium* (Bernardet & Bowman, 2006), but it is known that *Flavobacterium* can grow on it (Jooste et al., 1985). The preferred temperature varies inbetween the different species, but most *Flavobacterium* spp. grow at temperatures between $20 - 25\,^{\circ}\mathrm{C}$ which is considerably lower

than the temperatures applied in this study (Bernardet & Bowman, 2006). Theoretically, another reason may be, that *Flavobacterium* is not prevalent in the samples from the spinach-washing plant. This seems unlikely as *Flavobacterium* is widely distributed in aquatic habitats in the environment (Bernardet & Bowman, 2006).

4.7.4. Detection of *Arcobacter* by MALDI-TOF MS

To enable the detection of *Arcobacter* by MALDI-TOF MS selective media were used. Dilution series of all samples were prepared and plated on *Arcobacter* broth with 5 % horse blood with CAT and SSI and on Blood Agar plates. From the Blood Agar plates twelve colonies were isolated. Most of them were identified as *Pseudomonas* sp. by the SARAMIS software. Two colonies were not identified, but a possible match with *Arcobacter skirrowii* with a similarity of 40 % was found. 40 % is very low and usually not considered a correct match. The 16S rRNA gene of the colony was sequenced and analysis revealed the colonies to be *Tsukmarella* sp. and *Escherichia vulneris*. From the ASM-CAT plates three colonies were isolated none of which showed any similarity to *Arcobacter* according to the SARAMIS software. Four colonies were picked from the ASM-SS1 plates, two of which were matched with *Arcobacter butzleri* by the Saramis software. One of these two colonies was positively identified as *Arcobacter* sp. by sequence analysis.

These results show that an identification of *Arcobacter* or other bacteria is possible with MALDI-TOF MS, but it is essential to have suitable selective media and good reference spectra. The database contained only *Arcobacter skirrowii* and *A. butzleri* spectra at the time of the experiment. It is likely, that the comparison of one individual spectrum to another is more error prone than the comparison of an individual spectrum to a superspectrum. A superspectrum contains the most defining peaks from several strains of a species and therefore the strain-specific differences are balanced. This is not the case for *Arcobacter* as not many strains are known today and can be used in the construction of a superspectrum. MALDI-TOF MS may prove to be a very valuable tool for detection of pathogens in vegetable-processing and food control as it is a very simple, time- and resource-saving method which guarantees the viability of identified bacteria. Further experiments are necessary to establish the detection of *Arcobacter* by MALDI-TOF MS. The database has to be expanded to include spectra of all known species and of several isolates to ensure a solid base for reference.

5. Conclusion

Increasing numbers of produce-associated diseases of humans in the last years pose a challenge to producers of fruits and vegetables and to the public health agencies. New methods for an efficient and real-time control of the microbial load throughout the entire production chain of fresh vegetables and fruits have to be established. As a consequence, additional knowledge regarding the existence, growth, survival and infectious potential of enteric pathogens is required for a safer production and marketing of perishable foods.

In this study the microbial diversity of carrot wash water was established by a 16S rRNA gene clone library. Besides several pathogenic and opportunistic bacteria, *Arcobacter*, an emerging food pathogen was identified as the fourth most common genus. It is the first time that *Arcobacter* has been associated with vegetable-processing which was recently supported by the findings of González & Ferrús (2011).

PCR-Assays were developed to ascertain the occurrence of *Arcobacter* and *Pectobacterium* as an example for a phytopathogen in a spinach processing plant. Results obtained with qualitative PCR-Assays showed that both pathogens are present in the plant throughout the year regardless of disinfection methods and discontinuation of the production.

Especially *Arcobacter* was detected repeatedly in the wash basins. This lead to the assumption that *Arcobacter* may have colonized the plant probably forming a biofilm on the equipment and that uncontaminated vegetables may be contaminated during the washing process. In order to evaluate this assumption and to determine which *Arcobacter* species are prevalent in the plant, *Arcobacter*-specific 16S rRNA gene clone libraries were constructed.

Arcobacter butzleri and *A. cryaerophilus*, two pathogenic species, were identified alongside several environmental species and species that have not been described so far. Attempts to isolate these species were only successful in the case of *A. butzleri*. The other species could not be cultivated. However, this shows that pathogenic and viable *A. butzleri* cells are prevalent in the process line. The clone libraries also showed that the samples from the wash basins have a diversity distinct from the spinach samples and the sample of tap water. It could be seen that OTUs from the wash water were transferred onto the spinach during the wash process. This shows that a contamination during washing actually occurs. Furthermore, the microbial diversity of the wash water did not change over a period of time of two years. This suggests that *Arcobacter* colonized

the plant regardless of rigorous cleaning and disinfection procedures and the discontinuation of production during winter.

Recent epidemic events in Europe demonstrated a more efficient control of the production chain of fresh fruits and vegetables at regular intervals is essential. DNA-based methods represent valuable tools for the routine quality control, because of the speed of their application and their enhanced specificity compared to conventional microbiological identification methods.

In this study, new methods for the detection of *Arcobacter* and *Pectobacterium* in particular were developed. The aim was to develop a method which allows for a precise and fast identification of contaminating organisms during food processing. A multiplex-PCR was created which can distinguish nine *Arcobacter* species in a single PCR run. In combination with capillary electrophoresis this method proved to be highly accurate and sensitive. In future experiments the assay should be expanded to cover all known thirteen species. As new species are discovered it should be possible to add primer pairs for the new species to the assay. Samples of all kinds (solid, liquid, soil, blood, water) may be used as template. DNA-Isolation may not even be necessary in all cases. In this study enrichment procedures were required in order to detect *Arcobacter* species in the samples, since the numbers of cells were too low for a direct detection.

Quantitative PCR may provide the means for a control during the washing process or at another point in the processing of vegetables with the ability not only to identify the existing microorganisms, but also to quantify them. This will allow the producers of fresh fruits and vegetables to evaluate a potential risk for the consumers and consequently to realize batch-oriented processing steps. For *Arcobacter* and *Pectobacterium* qPCR-Assays were developed and tested on samples from the vegetable-washing plant. All assays successfully detected the pathogens with high accuracy. Since these assays are genus-specific they will provide a positive result for both pathogenic and non-pathogenic species. But in case of *Arcobacter* the occurrence of the non-pathogenic species may be an indicator for the occurrence of pathogenic species as well. The developed 23S rRNA gene based assay detected *Arcobacter* spp. which had been described at that time, this included pathogenic species which have been isolated most frequently in food processing.

Further experiments should focus on the development of PCR-based methods applicable to food processing which allow for a discrimination of living and dead cells. The lack of information on the viability of cells is a consistent limitation of PCR-based methods. One approach is to use Propidium monoadzide in combination with qPCR to distinguish between dead and viable cells of *Listeria monocytogenes* and *Bacillus subtilis* spores (Pan & Breidt, 2007, Rawsthorne et al., 2009).

In addition to solely DNA-based assays the potential of MALDI-TOF MS for ecologic studies and detection of pathogens was investigated. In contrast to the PCR-based methods MALDI-

TOF MS is a culture-based method. The diversity established with MALDI-TOF MS did not differ significantly from the diversity established with the 16S rRNA gene clone library. The major drawback of this method is the culture-dependent bias, but recent studies successfully explored the possibility of direct detection, thus circumventing the cultivation bias (Ferreira et al., 2011b,a, Kroumova et al., 2011). Detection of *Arcobacter* by MALDI-TOF MS was possible, but not reliable, since the spectra database only contained a limited number of reference spectra. MALDI-TOF MS depends on the existence of an extensive database that can be used for systematic comparison. In the future the existing database will be expanded so that robustness of the detection method will be improved.

All techniques developed and tested in this study were valuable and can be used for the detection of pathogens. However, a broader knowledge about the occurrence of pathogens in vegetable-processing facilities is still required. It is important to evaluate the route of transmission, to examine if the equipment itself is a source of contamination, and if so, what kind of procedures could prevent colonization of the equipment. This study discovered a considerable risk of vegetables being contaminated through the washing process.

Bibliography

Agric., U. D. (1965). *Losses in Agriculture*, vol. 291 of *USDA Handbook*. Agric. Res. Serv.

Amann, R., & Ludwig, W. (2000). Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. *FEMS Microbiology Reviews*, *24*(5), 555–565.

Amann, R. I., Ludwig, W., & Schleifer, K. H. (1995). Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. *Microbiological Reviews*, *59*(1), 143–169.

Anonymous (2002). Microbiology of food and animal feeding stuffs. polymerase chain reaction (PCR) for the detection of food-borne pathogens. general method specific requirements (EN ISO 22174). *International Organization for Standardization, Geneva, Switzerland*.

Anonymous (2011). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2009. *EFSA Journal*, *9*(3).

Ashelford, K. E., Chuzhanova, N. A., Fry, J. C., Jones, A. J., & Weightman, A. J. (2006). New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Applied and Environmental Microbiology*, *72*(9), 5734–5741.

Assanta, M. A., Roy, D., Lemay, M. J., & Montpetit, D. (2002). Attachment of *Arcobacter butzleri*, a new waterborne pathogen, to water distribution pipe surfaces. *Journal of Food Protection*, *65*(8), 1240–1247.

Atabay, H. I., Unver, A., Sahin, M., Otlu, S., Elmali, M., & Yaman, H. (2008). Isolation of various *Arcobacter* species from domestic geese *Anser anser*. *Veterinary Microbiology*, *128*(3-4), 400–405.

Babic, I., Roy, S., Watada, A. E., & Wergin, W. P. (1996). Changes in microbial populations on fresh cut spinach. *International Journal of Food Microbiology*, *31*(1-3), 107–119.

Bach, H. J., Jessen, I., Schloter, M., & Munch, J. C. (2003). A TaqMan-PCR protocol for quantification and differentiation of the phytopathogenic *Clavibacter michiganensis* subspecies. *Journal of Microbiological Methods*, *52*(1), 85–91.

Bae, W. K., Lee, Y. K., Cho, M. S., Ma, S. K., Kim, S. W., Kim, N. H., & Choi, K. C. (2006). A case of hemolytic uremic syndrome caused by *Escherichia coli* O104 : H4. *Yonsei Medical Journal*, *47*(3), 437–439.

Baggerman, W., & Koster, T. (1992). A comparison of enrichment and membrane filtration methods for the isolation of *Campylobacter* from fresh and frozen foods. *Food Microbiology*, *9*(2), 87 – 94.

Barbuddhe, S. B., Maier, T., Schwarz, G., Kostrzewa, M., Hof, H., Domann, E., Chakraborty, T., & Hain, T. (2008). Rapid identification and typing of *Listeria* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Applied and Environmental Microbiology*, *74*(17), 5402–5407.

Bastyns, K., Cartuyvels, D., Chapelle, S., Vandamme, P., Goossens, H., & DeWachter, R. (1995). A variable 23S rDNA region is a useful discriminating target for genus-specific and species-specific PCR amplification in *Arcobacter* species. *Systematic and Applied Microbiology*, *18*(3), 353–356.

Bej, A. K., Steffan, R. J., Dicesare, J., Haff, L., & Atlas, R. M. (1990). Detection of coliform bacteria in water by polymerase chain-reaction and gene probes. *Applied and Environmental Microbiology*, *56*(2), 307–314.

Bernardet, J. F., & Bowman, J. P. (2006). *The Prokaryotes: A Handbook on the Biology of Bacteria: Proteobacteria: Delta and Epsilon Subclasses. Deeply Rooted Bacteria*, vol. 7, chap. 6.4: The genus *Flavobacterium*, (pp. 481–532). Springer, Berlin, 3rd ed.

Beuchat, L. R. (1996). Pathogenic microorganisms associated with fresh produce. *Journal of Food Protection*, *59*(2), 204–216.

Beuchat, L. R. (2002). Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes and Infection*, *4*(4), 413 – 423.

Beuchat, L. R., & Ryu, J. H. (1997). Produce handling and processing practices. *Emerging Infectious Diseases*, *3*(4), 459–465.

Brightwell, G., Mowat, E., Clemens, R., Boerema, J., Pulford, D. J., & On, S. L. (2007). Development of a multiplex and real time PCR assay for the specific detection of *Arcobacter butzleri* and *Arcobacter cryaerophilus*. *Journal of Microbiological Methods*, *68*(2), 318–325.

Brooks, J. D., & Flint, S. H. (2008). Biofilms in the food industry: Problems and potential solutions. *International Journal Of Food Science And Technology*, *43*(12), 2163–2176.

Brouwer, M., Lievens, B., Van Hemelrijck, W., Van den Ackerveken, G., Cammue, B. P. A., & Thomma, B. P. H. J. (2003). Quantification of disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR. *FEMS Microbiology Letters*, *228*(2), 241–248.

Cain, T. C., Lubman, D. M., & Weber, W. J. (1994). Differentiation of bacteria using protein profiles from matrix-assisted laser-desorption ionization time-of-flight mass-spectrometry. *Rapid Communications In Mass Spectrometry*, *8*(12), 1026–1030.

Carlin, F., Nguyen-the, C., & Dasilva, A. A. (1995). Factors affecting the growth of *Listeria monocytogenes* on minimally processed fresh endive. *Journal of Applied Bacteriology*, *78*(6), 636–646.

Case, R. J., Boucher, Y., Dahllof, I., Holmstrom, C., Doolittle, W. F., & Kjelleberg, S. (2007). Use of 16S rRNA and *rpo*b genes as molecular markers for microbial ecology studies. *Applied and Environmental Microbiology*, *73*(1), 278–288.

CDC-Outbreak-Database (-).
URL `http://www.cdc.gov/foodborneoutbreaks/`

Cervenka, L., Kristlova, J., Peskova, I., Vytrasova, J., Pejchalova, M., & Brozkova, I. (2008). Persistence of *Arcobacter butzleri* CCUG 30484 on plastic, stainless steel and glass surfaces. *Brazilian Journal of Microbiology*, *39*(3), 517–520.

Chao, A. (1987). Estimating the population size for capture recapture data with unequal catchability. *Biometrics*, *43*(4), 783–791. J 249.

Christner, M., Rohde, H., Wolters, M., Sobottka, I., Wegscheider, K., & Aepfelbacher, M. (2010). Rapid identification of bacteria from positive blood culture bottles by use of matrix-assisted laser desorption-ionization time of flight mass spectrometry fingerprinting. *Journal Of Clinical Microbiology*, *48*(5), 1584–1591.

Cikos, S., & Koppel, J. (2009). Transformation of real-time PCR fluorescence data to target gene quantity. *Analytical Biochemistry*, *384*(1), 1–10.

Claydon, M. A., Davey, S. N., Edwards-Jones, V., & Gordon, D. B. (1996). The rapid identification of intact microorganisms using mass spectrometry. *Nature Biotechnology*, *14*(11), 1584–1586.

Collado, L., Inza, I., Guarro, J., & Figueras, M. J. (2008). Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. *Environmental Microbiology*, *10*(6), 1635–1640.

Crockett, C. S. (2007). The role of wastewater treatment in protecting water supplies against emerging pathogens. *Water Environment Research*, *79*(3), 221–232.

CSPI Outbreak database (-).
URL `http://www.cspinet.org/foodsafety/outbreak/pathogen.php`

Darrasse, A., Priou, S., Kotoujansky, A., & Bertheau, Y. (1994). PCR-based and restriction fragment length polymorphism of a *pel* gene as a tool to identify *Erwinia carotovora* in relation to potato diseases. *Applied and Environmental Microbiology*, *60*(5), 1437–1443.

de Hoffmann, E., & Stroobant, V. (2007). *Mass spectrometry: Principles and applications*, chap. 2.4 Time-of-Flight Analysers, (pp. 126–142). Wiley-Interscience.

De Roever, C. (1998). Microbiological safety evaluations and recommendations on fresh produce. *Food Control*, *9*(6), 321–347.

DeWaal, C. S., Hicks, G., Barlow, K., Alderton, L., & Vegosen, L. (2006). Foods associated with foodborne illness outbreaks from 1990 through 2003. *Food Protection Trends*, *26*(7), 466–473.

DeWaal, C. S., Tian, X. A., & Plunkett, D. (2009). Outbreak alert 2009. *Center for science in the public interest (CSPI)*.
URL `http://www.cspinet.org/foodsafety/outbreak_report_2009.html`

Dieckmann, R., Helmuth, R., Erhard, M., & Malorny, B. (2008). Rapid classification and identification of *Salmonellae* at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Applied and Environmental Microbiology*, *74*(24), 7767–7778.

Donachie, S., Foster, J., & Brown, M. (2007). Culture clash: Challenging the dogma of microbial diversity. *ISME J*, *1*(2), 97–99.

Donohue, M. J., Smallwood, A. W., Pfaller, S., Rodgers, M., & Shoemaker, J. A. (2006). The development of a matrix-assisted laser desorption/ionization mass spectrometry-based method for the protein fingerprinting and identification of *Aeromonas* species using whole cells. *Journal of Microbiological Methods*, *65*(3), 380–389.

Douidah, L., De Zutter, L., Vandamme, P., & Houf, K. (2010). Identification of five human and mammal associated *Arcobacter* species by a novel multiplex-PCR assay. *Journal of Microbiological Methods*, *80*(3), 281–286.

Edwards-Jones, V., Claydon, M. A., Evason, D. J., Walker, J., Fox, A. J., & Gordon, D. B. (2000). Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. *Journal of Medical Microbiology*, *49*(3), 295–300.

EFSA (2011a). Shiga toxin-producing *E. coli* (STEC): Update on outbreak in the EU (15 july 2011, 11:00). *press release*, *July 15*.

EFSA (2011b). Tracing seeds, in particular fenugreek (*Trigonella foenum-graecum*) seeds, in relation to the shiga toxin-producing *E. coli* (STEC) O104:h4 2011 outbreaks in Germany and France. *Technical Report of EFSA*.

EstimateS (-).
URL `http://viceroy.eeb.uconn.edu/EstimateS`

Fagerquist, C. K., Bates, A. H., Heath, S., King, B., Garbus, B. R., Harden, L. A., & Miller, W. (2006). Sub-speciating *Campylobacter jejuni* by proteomic analysis of its protein biomarkers and their post-translational modifications. *Journal of Proteome Research*, *5*(10), 2527–2538.

Felsenstein, J. (1985). Confidence-limits on phylogenies - an approach using the bootstrap. *Evolution*, *39*(4), 783–791.

Fera, M. T., Maugeri, T. L., Gugliandolo, C., Beninati, C., Giannone, M., La Camera, E., & Carbone, M. (2004). Detection of *Arcobacter* spp. in the coastal environment of the Mediterranean Sea. *Applied and Environmental Microbiology*, *70*(3), 1271–1276.

Ferreira, L., Sanchez-Juanes, F., Munoz-Bellido, J. L., & Gonzalez-Buitrago, J. M. (2011a). Rapid method for direct identification of bacteria in urine and blood culture samples by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: intact cell vs. extraction method. *Clinical Microbiology and Infection*, *17*(7), 1007–1012.

Ferreira, L., Sanchez-Juanes, F., Porras-Guerra, I., Garcia-Garcia, M. I., Garcia-Sanchez, J. E., Gonzalez-Buitrago, J. M., & Munoz-Bellido, J. L. (2011b). Microorganisms direct identification from blood culture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Clinical Microbiology And Infection*, *17*(4), 546–551.

Figueras, M. J., Collado, L., & Guarro, J. (2008). A new 16S rDNA-RFLP method for the discrimination of the accepted species of *Arcobacter*. *Diagnostic Microbiology and Infectious Disease*, *62*(1), 11–15. J 0.

Food and Agriculture organization (-). Post-harvest losses aggravate hunger.
URL `http://www.fao.org/news/story/en/item/36844/icode/`

Franz, E., & van Bruggen, A. H. (2008). Ecology of *E. coli* O157:h7 and *Salmonella enterica* in the primary vegetable production chain. *Critical Reviews in Microbiology*, *34*(3-4), 143–161.

Fröhlingsdorf, M., Hackenbroch, V., Ludwig, U., & Thadeusz, F. (2011). Unrat im Wasser. *Der Spiegel*, *26*, 130–131.

Friedrichs, C., Rodloff, A. C., Chhatwal, G. S., Schellenberger, W., & Eschrich, K. (2007). Rapid identification of viridans streptococci by mass spectrometric discrimination. *Journal of Clinical Microbiology*, *45*(8), 2392–2397.

Gilbride, K. A., Lee, D. Y., & Beaudette, L. A. (2006). Molecular techniques in wastewater: Understanding microbial communities, detecting pathogens, and real-time process control. *Journal of Microbiological Methods*, *66*(1), 1–20. J JUL.

Girones, R., Ferrus, A. M., Alonso, J. L., Rodriguez-Manzano, J., Calgua, B., de Abreu Correa, A., Hundesa, A., Carratala, A., & Bofill-Mas, S. (2010). Molecular detection of pathogens in water - The pros and cons of molecular techniques. *Water Research*, *44*(15), 4325–4339.

González, A., & Ferrús, M. (2011). Study of *Arcobacter* spp. contamination in fresh lettuces detected by different cultural and molecular methods. *International Journal of Food Microbiology*, *145*(1), 311–314.

González, A., Suski, J., & Ferrús, M. A. (2010). Rapid and accurate detection of *Arcobacter* contamination in commercial chicken products and wastewater samples by real-time polymerase chain reaction. *Foodborne Pathogens and Disease*, *7*(3), 327–338.

Good, I. J. (1953). The population frequencies of species and the estimation of population parameters. *Biometrika*, *40*(3-4), 237–264.

Gugliandolo, C., Irrera, G. P., Lentini, V., & Maugeri, T. L. (2008). Pathogenic *Vibrio*, *Aeromonas* and *Arcobacter* spp. associated with copepods in the Straits of Messina (Italy). *Marine Pollution Bulletin*, *56*(3), 600–606.

Hammer, O., Harper, D. A. T., & Ryan, P. D. (2001). PAST: palaeontological statistics software package for education and data analysis. *Palaeontologia Electronica*, *4*, 9–11.

Hedberg, C. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases*, *5*(6), 840–841.

Hedberg, C. W., MacDonald, K. L., & Osterholm, M. T. (1994). Changing epidemiology of food-borne disease: A Minnesota perspective. *Clinical Infectious Diseases*, *18*(5), 671–680.

Heid, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (1996). Real time quantitative PCR. *Genome Research*, *6*(10), 986–994.

Higuchi, R., Fockler, C., Dollinger, G., & Watson, R. (1993). Kinetic PCR-DGGE analysis - real-time monitoring of DNA-amplification reactions. *Biotechnology*, *11*(9), 1026–1030.

Ho, H. T. K., Lipman, L. J. A., & Gaastra, W. (2008). The introduction of *Arcobacter* spp. in poultry slaughterhouses. *International Journal of Food Microbiology*, *125*(3), 223–229.

Holland, R. D., Wilkes, J. G., Rafii, F., Sutherland, J. B., Persons, C. C., Voorhees, K. J., & Lay, J. O. (1996). Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. *Rapid Communications In Mass Spectrometry*, *10*(10), 1227–1232.

Holland, S. (-). Rarefaction v1.3.
URL `http://www.uga.edu/strata/software/`

Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., & Fach, P. (2004). Practical considerations in design of internal amplification controls for diagnostic PCR assays. *Journal of Clinical Microbiology*, *42*(5), 1863–1868.

Houf, K., De Zutter, L., Van Hoof, J., & Vandamme, P. (2002). Assessment of the genetic diversity among Arcobacters isolated from poultry products by using two PCR-based typing methods. *Applied and Environmental Microbiology*, *68*(5), 2172–2178.

Houf, K., Devriese, L. A., De Zutter, L., Van Hoof, J., & Vandamme, P. (2001). Development of a new protocol for the isolation and quantification of *Arcobacter* species from poultry products. *International Journal of Food Microbiology*, *71*(2-3), 189–196.

Houf, K., Tutenel, A., De Zutter, L., Van Hoof, J., & Vandamme, P. (2000). Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*. *FEMS Microbiology Letters*, *193*(1), 89–94.

Hudson, A., & Turner, N. (2002). Risks associated with bacterial pathogens in exported fruit and vegetables. *New Zealand Food Safety Authority*.

Hughes, J. B., Hellmann, J. J., Ricketts, T. H., & Bohannan, B. J. M. (2001). Counting the uncountable: Statistical approaches to estimating microbial diversity. *Applied and Environmental Microbiology*, *67*(10), 4399–4406.

Ilina, E. N., Borovskaya, A. D., Malakhova, M. M., Vereshchagin, V. A., Kubanova, A. A., Kruglov, A. N., Svistunova, T. S., Gazarian, A. O., Maier, M., T. Kostrzewa, & Govorun, V. M. (2009). Direct bacterial profiling by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry for identification of pathogenic *Neisseria*. *Journal of Molecular Diagnostics*, *11*(1), 75–86.

Ilina, E. N., Borovskaya, A. D., Serebryakova, M. V., Chelysheva, V. V., Momynaliev, K. T., Maier, T., Kostrzewa, M., & Govorun, V. M. (2010). Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the study of *Helicobacter pylori*. *Rapid Communications in Mass Spectrometry*, *24*(3), 328–334.

Jacob, J., Lior, H., & Feuerpfeil, I. (1993). Isolation of *Arcobacter butzleri* from a drinking-water reservoir in Eastern Germany. *Zentralblatt fur Hygiene und Umweltmedizin*, *193*(6), 557–562.

Jacob, J., Woodward, D., Feuerpfeil, I., & Johnson, W. M. (1998). Isolation of *Arcobacter butzleri* in raw water and drinking water treatment plants in Germany. *Zentralblatt fur Hygiene und Umweltmedizin*, *201*(2), 189–198.

Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A., & Uyttendaele, M. (2010). Alternative microbial methods: An overview and selection criteria. *Food Microbiology*, *27*(6), 710–730.

Jensen, M. A., Webster, J. A., & Straus, N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA-spacer polymorphisms. *Applied and Environmental Microbiology*, *59*(4), 945–952.

Jooste, P. J., Britz, T. J., & Dehaast, J. (1985). A numerical taxonomic study of *Flavobacterium cytophaga* strains from dairy sources. *Journal of Applied Bacteriology*, *59*(4), 311–323.

Josephson, K., Gerba, C., & Pepper, I. (1993). Polymerase chain reaction detection of nonviable bacterial pathogens. *Applied And Environmental Microbiology*, *59*(10), 3513–3515.

Karas, M., & Hillenkamp, F. (1988). Laser desorption ionization of proteins with molecular masses exceeding 10000 daltons. *Analytical Chemistry*, *60*(20), 2299–2301.

Karch, H., Tarr, P. I., & Bielaszewska, M. (2005). Enterohaemorrhagic *Escherichia coli* in human medicine. *Int J Med Microbiol*, *295*(6-7), 405–418.

Kawasaki, S., Fratamico, P. M., Horikoshi, N., Okada, Y., Takeshita, K., Sameshima, T., & Kawamoto, S. (2009). Evaluation of a multiplex PCR system for simultaneous detection of *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:h7 in foods and in food subjected to freezing. *Foodborne Pathogens And Disease*, *6*(1), 81–89.

Keswani, J., & Whitman, W. B. (2001). Relationship of 16S rRNA sequence similarity to DNA-hybridization in prokaryotes. *International Journal of Systematic and Evolutionary Microbiology*, *51*, 667–678.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*(2), 111–120. J 6320.

Kirk, J., Beaudette, L., Hart, M., Moutoglis, P., Khironomos, J., Lee, H., & Trevors, J. (2004). Methods of studying soil microbial diversity. *Journal Of Microbiological Methods*, *58*(2), 169–188.

Kleparnik, K., & Bocek, P. (2007). DNA diagnostics by capillary electrophoresis. *Chemical Reviews*, *107*(11), 5279–5317.

Klocke, M., Mähnert, P., Mundt, K., Souidi, K., & Linke, B. (2007). Microbial community analysis of a biogas-producing completely stirred tank reactor fed continuously with fodder beet silage as mono-substrate. *Systematic and Applied Microbiology*, *30*(2), 139–151.

Kolaczkowski, B., & Thornton, J. W. (2004). Performance of maximum parsimony and likelihood phylogenetics when evolution is heterogeneous. *Nature*, *431*(7011), 980–984.

Kroumova, V., Gobbato, E., Basso, E., Mucedola, L., Giani, T., & Fortina, G. (2011). Direct identification of bacteria in blood culture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: A new methodological approach. *Rapid communications in mass spectrometry : RCM*, *25*(15), 2247–9.

Lane, D. J. (1991). *Nucleic acid techniques in bacterial systematics.*, chap. 16S/23S rRNA sequencing, (pp. 115–175). Wiley and Sons.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences of the United States of America*, *82*(20), 6955–6959.

Lartigue, M. F., Hery-Arnaud, G., Haguenoer, E., Domelier, A. S., Schmit, P. O., van der Mee-Marquet, N., Lanotte, P., Mereghetti, L., Kostrzewa, M., & Quentin, R. (2009). Identification of *Streptococcus agalactiae* isolates from various phylogenetic lineages by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology*, *47*(7), 2284–2287.

Leuschner, R. G. K., Beresford-Jones, N., & Robinson, C. (2004). Difference and consensus of whole cell *Salmonella enterica* subsp. *enterica* serovars matrix-assisted laser desorption/ionization time-of-flight mass spectrometry spectra. *Letters In Applied Microbiology*, *38*(1), 24–31.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., & Schleifer, K.-H. (2004). ARB: A software environment for sequence data. *Nucleic Acids Research*, *32*(4), 1363–1371.

Mackay, I., Arden, K., & Nitsche, A. (2002). Real-time PCR in virology. *Nucleic Acids Research*, *30*(6), 1292–1305.

Mackay, I. M. (2004). Real-time PCR in the microbiology laboratory. *Clinical Microbiology and Infection*, *10*(3), 190–212.

Malorny, B., Tassios, P., Radstrom, P., Cook, N., Wagner, M., & Hoorfar, J. (2003). Standardization of diagnostic PCR for the detection of foodborne pathogens. *International Journal Of Food Microbiology*, *83*(1), 39–48.

Mamyrin, B. (1994). Laser-assisted reflectron time-of-flight mass-spectrometry. *International Journal Of Mass Spectrometry And Ion Processes*, *131*, 1–19.

Masters, C., Shallcross, J., & Mackey, B. (1994). Effect of stress treatments on the detection of *Listeria Monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain-reaction. *Journal Of Applied Bacteriology*, *77*(1), 73–79.

McNaught, A. D., & Wilkinson, A. (1997). *IUPAC. Compendium of Chemical Terminology*, vol. 2nd. Oxford: Blackwell Scientific Publications.
URL `http://goldbook.iupac.org`

Mead, P., Slutsker, L., Dietz, V., McCaig, L., Bresee, J., Shapiro, C., Griffin, P., & Tauxe, R. (2000). Food-related illness and death in the United States (reprinted from emerging infectious diseases). *Journal Of Environmental Health*, *62*(7), 9–18.

Mellmann, A., Harmsen, D., Harmsen, D., Cummings, C. A., Zentz, E. B., Leopold, S. R., Rico, A., Prior, K., Szczepanowski, R., Ji, Y., Zhang, W., McLaughlin, S., Henkhaus, J. K., Leopold, B., Bielaszewska, M., Prager, R., Brzoska, P. M., Moore, R. L., Guenther, S., Rothberg, J., &

Karch, H. (2011). Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:h4 outbreak by rapid next generation sequencing technology. *PLoS One*, *6*(7). E22751.

Mocak, J., Bond, A. M., Mitchell, S., & Scollary, G. (1997). A statistical overview of standard (IUPAC and ACS) and new procedures for determining the limits of detection and quantification: Application to voltammetric and stripping techniques (technical report). *Pure and Applied Chemistry*, *69*(2), 297–328.

Moreira, D. (1998). Efficient removal of PCR inhibitors using agarose-embedded DNA preparations. *Nucleic Acids Research*, *26*(13), 3309–3310.

Mullis, K. B., & Faloona, F. A. (1987). Specific synthesis of DNA invitro via a polymerase-catalyzed chain-reaction. *Methods In Enzymology*, *155*, 335–350.

Munoz, R., Lopez-Lopez, A., Urdiain, M., Moore, E. R. B., & Rossello-Mora, R. (2011). Evaluation of matrix-assisted laser desorption ionization-time of flight whole cell profiles for assessing the cultivable diversity of aerobic and moderately halophilic prokaryotes thriving in solar saltern sediments. *Systematic and Applied Microbiology*, *34*(1, Sp. Iss. SI), 69–75.

Murray, M. G., & Thompson, W. F. (1980). Rapid isolation of high molecular-weight plant DNA. *Nucleic Acids Research*, *8*(19), 4321–4325. J 2841.

NCBI-Database (-).
URL `http://www.ncbi.nlm.nih.gov`

Neuwirth, E. (2008). R meets the workplace - embedding R in Excel to make it more accessible. *March 31*.

Nguyen-the, C., & Carlin, F. (1994). The microbiology of minimally processed fresh fruits and vegetables. *Critical reviews in food science and nutrition*, *34*(4), 371–401.

Oliver, J. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews*, *34*(4), 415–425.

Olsen, S., Linda, C., MacKinon, M., Goulding, J., Bean, N., & Slutsker, L. (2000). *Surveillance for Foodborne Disease Outbreaks –United States, 1993-1997*, vol. 49(SS01) of *Morbidity and Mortality Weekly Report*. Centers for Disease Control and Prevention.

On, S. L. W., Atabay, H. I., Amisu, K. O., Coker, A. O., & Harrington, C. S. (2004). Genotyping and genetic diversity of *Arcobacter butzleri* by amplified fragment length polymorphism (AFLP) analysis. *Letters in Applied Microbiology*, *39*(4), 347–352. J Article.

On, S. L. W., Harrington, C. S., & Atabay, H. I. (2003). Differentiation of *Arcobacter* species by numerical analysis of AFLP profiles and description of a novel *Arcobacter* from pig abortions and turkey faeces. *Journal of Applied Microbiology*, *95*(5), 1096–1105.

On, S. L. W., Jensen, T. K., Bille-Hansen, V., Jorsal, S. E., & Vandamme, P. (2002). Prevalence and diversity of *Arcobacter* spp. isolated from the internal organs of spontaneous porcine abortions in Denmark. *Veterinary Microbiology*, *85*(2), 159–167.

on Microbiological Criteria for Foods, N. A. C. (1999). Microbiological safety evaluations and recommendations on sprouted seeds. *International Journal of Food Microbiology*, *52*, 123–153.

Pan, Y., & Breidt, F. J. (2007). Enumeration of viable *Listeria monocytogenes* cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. *Applied and Environmental Microbiology*, *73*(24), 8028–8031.

Payasi, A., Sanwal, R., & Sanwal, G. G. (2009). Microbial pectate lyases: characterization and enzymological properties. *World Journal of Microbiology & Biotechnology*, *25*(1), 1–14.

Perera, M. R., Vanstone, V. A., & Jones, M. G. K. (2005). A novel approach to identify plant parasitic nematodes using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications In Mass Spectrometry*, *19*(11), 1454–1460.

Perombelon, M. C. M. (2002). Potato diseases caused by soft rot erwinias: an overview of pathogenesis. *Plant Pathology*, *51*(1), 1–12.

Pfaffl, M. W., & Hageleit, M. (2001). Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. *Biotechnology Letters*, *23*(4), 275–282.

Phillips, C. A. (2001). Arcobacters as emerging human foodborne pathogens. *Food Control*, *12*(1), 1–6. J Review JAN.

Pospiech, A., & Neumann, B. (1995). A versatile quick-prep of genomic DNA from Gram-positive bacteria. *Trends In Genetics*, *11*(6), 217–218.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., & Glöckner, F. O. (2007). Silva: a comprehensive online resource for quality checked and aligned ribosomal rna sequence data compatible with arb. *Nucleic Acids Res*, *35*(21), 7188–7196.
URL `http://dx.doi.org/10.1093/nar/gkm864`

Pruitt, T. T., K. D., & Maglott, D. R. (2007). Ncbi reference sequences (refseq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Research*, *35*, D61–D65. J Article JAN Sp. Iss. SI.
URL `ISI:000243494600014`

Rawsthorne, H., Dock, C. N., & Jaykus, L. A. (2009). PCR-based method using propidium monoazide to distinguish viable from nonviable *Bacillus subtilis* spores. *Applied and Environmental Microbiology*, *75*(9), 2936–2939.

Rheims, H., & Stackebrandt, E. (1999). Application of nested polymerase chain reaction for the detection of as yet uncultured organisms of the class Actinobacteria in environmental samples. *Environmental Microbiology*, *1*(2), 137–143.

Rice, E. W., Rodgers, M. R., Wesley, I. V., Johnson, C. H., & Tanner, S. A. (1999). Isolation of *Arcobacter butzleri* from ground water. *Letters in Applied Microbiology*, *28*(1), 31–35.

Rodriguez-Lazaro, D., Lombard, B., Smith, H., Rzezutka, A., D'Agostino, M., Helmuth, R., Schroeter, A., Malorny, B., Miko, A., Guerra, B., Davison, J., Kobilinsky, A., Hernandez, M., Bertheau, Y., & Cook, N. (2007). Trends in analytical methodology in food safety and quality: monitoring microorganisms and genetically modified organisms. *Trends in food science & technology*, *18*(6), 306–319.

Rosmini, M. R., Signorini, M. L., Schneider, R., & Bonazza, J. C. (2004). Evaluation of two alternative techniques for counting mesophilic aerobic bacteria in raw milk. *Food Control*, *15*(1), 39–44.

Saitou, N., & Nei, M. (1987). The Neighbor-joining method - a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425.

Sapers, G. M. (2001). Efficacy of washing and sanitizing methods for disinfection of fresh fruit and vegetable products. *Food Technology and Biotechnology*, *39*(4), 305–311.

Sekiguchi, Y., Kamagata, Y., Syutsubo, K., Ohashi, A., Harada, H., & Nakamura, K. (1998). Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16S rRNA gene analysis. *Microbiology-Sgm*, *144*, 2655–2665.

Sipos, R., Szekely, A. J., Palatinszky, M., Revesz, S., Marialigeti, K., & Nikolausz, M. (2007). Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis. *Fems Microbiology Ecology*, *60*(2), 341–350.

Son, I., Englen, M. D., Berrang, M. E., Fedorka-Cray, P. J., & Harrison, M. A. (2007). Prevalence of *Arcobacter* and *Campylobacter* on broiler carcasses during processing. *International Journal of Food Microbiology*, *113*(1), 16–22.

Stampi, S., De Luca, G., Varoli, O., & Zanetti, F. (1999). Occurrence, removal and seasonal variation of thermophilic *Campylobacter* and *Arcobacter* in sewage sludge. *Zentralblatt fur Hygiene und Umweltmedizin*, *202*(1), 19–27.

Stampi, S., Varoli, O., Zanetti, F., & DeLuca, G. (1993). *Arcobacter cryaerophilus* and thermophilic *Campylobacter* in a sewage treatment plant in Italy - 2 secondary treatments compared. *Epidemiology and Infection*, *110*(3), 633–639.

Struelens, M. J., Palm, D., & Takkinen, J. (2011). Enteroaggregative, Shiga toxin-producing *Escherichia coli* O104:h4 outbreak: new microbiological findings boost coordinated investigations by European public health laboratories. *Eurosurveillance*, *16*(24), 2–4.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, *24*(8), 1596–1599.

Tanigawa, K., Kawabata, H., & Watanabe, K. (2010). Identification and typing of *Lactococcus lactis* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Applied and Environmental Microbiology*, *76*(12), 4055–4062.

The Commission of the European Communities (2005). Commission regulation (EC) No 2073/2005 of 15 november 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union*, (pp. L 338/1–L 338/26).

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). Clustal-W - improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*(22), 4673–4680.

Toth, I. K., Avrova, A. O., & Hyman, L. J. (2001). Rapid identification and differentiation of the soft rot erwinias by 16s-23s intergenic transcribed spacer-pcr and restriction fragment length polymorphism analyses. *Applied and Environmental Microbiology*, *67*(9), 4070–4076. J SEP.

Toth, I. K., Bell, K. S., Holeva, M. C., & Birch, P. R. J. (2003). Soft rot Erwiniae: from genes to genomes. *Molecular Plant Pathology*, *4*(1), 17–30.

van Driessche, E., & Houf, K. (2007). Discrepancy between the occurrence of *Arcobacter* in chickens and broiler carcass contamination. *Poultry Science*, *86*(4), 744–751.

van Driessche, E., Houf, K., Vangroenweghe, F., De Zutter, L., & van Hoof, J. (2005). Prevalence, enumeration and strain variation of *Arcobacter* species in the faeces of healthy cattle in Belgium. *Veterinary Microbiology*, *105*(2), 149–154.

van Driessche, E., Houf, K., Vangroenweghe, F., Nollet, N., De Zutter, L., Vandamme, P., & van Hoof, J. (2004). Occurrence and strain diversity of *Arcobacter* species isolated from healthy Belgian pigs. *Research in Microbiology*, *155*(8), 662–666.

von Wintzingerode, F., Gobel, U. B., & Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews*, *21*(3), 213–229.

Vorob'eva, L. I., Khasaeva, F. M., Vasilyuk, N. V., & Trenquil, E. (2011). Characterization of propionic acid bacteria using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. *Microbiology*, *80*(5), 664–671.

Walker, J., Fox, A. J., Edwards-Jones, V., & Gordon, D. B. (2002). Intact cell mass spectrometry (ICMS) used to type methicillin-resistant *Staphylococcus aureus*: media effects and inter-laboratory reproducibility. *Journal of Microbiological Methods*, *48*(2-3), 117–126.

Wang, M., Ahrne, S., Jeppsson, B., & Molin, G. (2005). Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. *Fems Microbiology Ecology*, *54*(2), 219–231.

Wang, S. S., & Levin, R. E. (2006). Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. *Journal Of Microbiological Methods*, *64*(1), 1–8.

Wells, J. M., & Butterfield, J. E. (1997). *Salmonella* contamination associated with bacterial soft rot of fresh fruits and vegetables in the marketplace. *Plant Disease*, *81*(8), 867–872.

Wells, J. M., & Butterfield, J. E. (1999). Incidence of *Salmonella* on fresh fruits and vegetables affected by fungal rots or physical injury. *Plant Disease*, *83*(8), 722–726.

Wilson, C. L., & Wisniewski, M. E. (1989). Biological control of postharvest diseases of fruits and vegetables: an emerging technology. *Annual Review of Phytopathology*, *27*, 425–441.

Woese, C. R. (1987). Bacterial evolution. *Microbiological Reviews*, *51*(2), 221–271.

Yap, M. N., Barak, J. D., & Charkowski, A. O. (2004). Genomic diversity of *Erwinia carotovora* subsp. *carotovora* and its correlation with virulence. *Applied and Environmental Microbiology*, *70*(5), 3013–3023.

Yarza, P., Richter, M., Peplies, J., Euzeby, J., Amann, R., Schleifer, K. H., Ludwig, W., Glöckner, F. O., & Rossello-Mora, R. (2008). The All-Species living tree project: A 16S rRNA-based phylogenetic tree of all sequenced type strains. *Systematic and Applied Microbiology*, *31*, 241–250.

A. List of publications

A.1. Publications

Hausdorf L, Fröhling A, Schlüter O, Klocke M, Adamzig H, Walter AD; Hygieneüberwachung per Chip: Prozessbegleitende Detektion von human- und phytopathogenen Mikroorganismen bei der Aufbereitung von Frischeprodukten. (2008) (Hygiene monitoring per chip: detecting human- and phytopathogens during post harvest vegetable processing. Internet-Publikation. URL: http://www.landtechnik-net.com). Landtechnik 63 (4): 224-225.

Hausdorf L, Fröhling A, Schlüter O, Klocke M; Analysis of the bacterial community within carrot wash water. (2011) Canadian Journal of Microbiology 57:(5), 447-452.

Hausdorf L, Fröhling A, Bergmann I, Sobiella K, Mundt K, Schlüter O, Klocke M; Enumeration of *Arcobacter* sp. in vegetable processing plants and evaluation of its genetic diversity. Journal of food protection (in preparation)

Hausdorf L, Rheinhardt S, Fröhling A, Schlüter O, Klocke M; Detection of *Arcobacter* sp. by multiplex-PCR and capillary-electrophoresis. Food and Bioprocess Technology (in preparation)

Hausdorf L, Winzer M, Mundt K, Fröhling A, Schlüter O, Cordes C, Klocke M; Evaluation of microbial diversity of vegetable wash water by MALDI-TOF MS Environmental Microbiology (in preparation)

A.2. Proceedings

Hausdorf L, Fröhling A, Nettmann E, Schlüter O, Klocke M; Detection of *Arcobacter* during processing of vegetables. (2008) Conference proceedings CD: 1176926. International Conference on Agricultural Engineering (AgEng), June 23rd – 25th, Crete, Greece (poster presentation).

A.3. Poster and oral presentations

Hausdorf L, Fröhling A, Mundt, K, Schlüter O, Klocke M; Genetic diversity of *Arcobacter* spp. in a vegetable-processing plant. (2011) 4th Congress of European Microbiologists FEMS 2011, June 25 - 30, Geneva, Switzerland (poster presentation).

Hausdorf L.; Occurence of *Arcobacter* sp. in vegetable processing plants. (2010) BerlinFOOD 2010, Sep. 8th - 10th, Berlin, Germany (oral presentation).

Fröhling A, Adamzig H, Walter AD, Hausdorf L, Klocke M, Schlüter O; Biosensors for the detection of pathogenic microorganisms – Concepts for determination of pathogens in fruits and vegetable processing using PCR-techniques and flow cytometry. (2008) ProSenso.net2 Workshop at the Post harvest unlimited, Nov. 4th – 7th, Potsdam, Germany (oral presentation).

Schlüter O, Adamzig H, Walter AD, Fröhling A, Hausdorf L, Klocke M; Microtechnology for in-situ detection of pathogens during post harvest processing of vegetables. (2008) 10th International Congress of Engineering and Food, April 20th – 24th, Viña del Mar, Chile (oral presentation).

Walter AD, Mertsch O, Adamzig H, Fröhling A, Hausdorf L, Klocke S, Klocke M, Schlüter O, Schondelmaier D, Loechel B; Contamination Control of Agricultural Products by On- Chip PCR and Flow Cytometry. (2007) 12th International Commercialization of Micro and Nano Systems Conference, Sep. 2nd - 6th, Melbourne, Australia (oral presentation).

B. Material

Table B.1.: Chemical substances used in this study.

Chemical substance	Manufacturer
Ampicillin	AppliChem GmbH, Darmstadt
Antifading-solution Citifluor	PLANO, Wetzlar
ROTISOLV HPLC Gradient Grade water	Carl Roth GmbH & Co.KG, Karlsruhe
Biozym Phor Agarose	Biozym Scientific GmbH, Hessisch Oldendorf
Biozym LE Agarose	Biozym Scientific GmbH, Hessisch Oldendorf
Agarose Low Melt 3	AppliChem GmbH, Darmstadt
Bromphenol blue	AppliChem GmbH, Darmstadt
Calcium chloride	AppliChem GmbH, Darmstadt
Membranefilter Type 111 (0.45 µm)	VWR International GmbH, Darmstadt
Chloroform	AppliChem GmbH, Darmstadt
CTAB	AppliChem GmbH, Darmstadt
DAPI	Carl Roth GmbH & Co.KG, Karlsruhe
EDTA	AppliChem GmbH, Darmstadt
Acetic acid	AppliChem GmbH, Darmstadt
Ethanol	AppliChem GmbH, Darmstadt
Ethidium bromide 1 % (0.01 g/ml) in water	SERVA Electrophoresis GmbH, Heidelberg
Formamide	AppliChem GmbH, Darmstadt
Glycerol	AppliChem GmbH, Darmstadt
Hydrochloric acid	AppliChem GmbH, Darmstadt
IPTG	Carl Roth GmbH & Co.KG, Karlsruhe
Isoamyl alcohol	AppliChem GmbH, Darmstadt
Isopropyl alcohol	AppliChem GmbH, Darmstadt
Calf thymus-DNA	Sigma-Aldrich Chemie GmbH, München
Calium chloride	AppliChem GmbH, Darmstadt
$MgCl_2$, 25 mM	Fermentas GmbH, St. Leon-Rot
dNTP-Mix (10 mM each)	Fermentas GmbH, St. Leon-Rot

Chemical substance	Manufacturer
Taq-Polymerase	Fermentas GmbH, St. Leon-Rot
10 x Taq-Buffer with $(NH_4)2SO_4$	Fermentas GmbH, St. Leon-Rot
Sample loading solution (SLS)	Beckman Coulter GmbH, Krefeld
Potassium dihydrogen phophate	AppliChem GmbH, Darmstadt
LB-Agar	Carl Roth GmbH & Co.KG, Karlsruhe
Lysozyme	AppliChem GmbH, Darmstadt
Sodium chloride	Carl Roth GmbH & Co.KG, Karlsruhe
SYBR®GREEN PCR mastermix	Applied Biosystems GmbH, Darmstadt
MaximaTM SYBR-Green/ROX qPCR Master Mix (2x)	Fermentas GmbH, St. Leon-Rot
MaximaTM Probe qPCR Master Mix (2x)	Fermentas GmbH, St. Leon-Rot
Disodium hydrogen phosphate	AppliChem GmbH, Darmstadt
Quant-iT TMPicoGreen® dsDNA (in DMSO)	Invitrogen GmbH, Darmstadt
Proteinase K	AppliChem GmbH, Darmstadt
Polyvinylpolypyrrolidone (PVPP)	AppliChem GmbH, Darmstadt
Sodium dodecly sulfate	AppliChem GmbH, Darmstadt
1 x TE-Buffer *pH* 8.0	AppliChem GmbH, Darmstadt
TRIS	AppliChem GmbH, Darmstadt
X-Galactose	Carl Roth GmbH & Co.KG, Karlsruhe
Trimethoprim	Sigma-Aldrich GmbH, München
5-Fluorouracil	Sigma-Aldrich GmbH, München
Amphotericin B	Sigma-Aldrich GmbH, München
Cefoperazone	Sigma-Aldrich GmbH, München
Novobiocin	Sigma-Aldrich GmbH, München

Table B.4.: Commercial kits used in this study

Kit	Manufacturer
QIAquick PCR Purification Kit	Qiagen, Hilden
NucleoSpin Plasmid DNA Purification Kit	Macherey-Nagel, Düren
pGEM-T Easy Vector System II Kit	Promega GmbH, Mannheim
FastDNA Spin Kit for soil	MP Biomedicals, Heidelberg

Table B.2.: Media used in this study

Media	Manufacturer
Standard I Agar	Carl Roth GmbH & Co.KG, Karlsruhe
Arcobacter-Broth (CM 965)	Oxoid Limited, Hampshire, UK
Brain Hearth Infusion	Oxoid Limited, Hampshire, UK
Nutrient broth	Carl Roth GmbH & Co.KG, Karlsruhe
McConkey Agar	Carl Roth GmbH & Co.KG, Karlsruhe
Chromocult Agar	Merck KGaA, Darmstadt
Marine Agar	Carl Roth GmbH & Co.KG, Karlsruhe
LB-Medium (Lennox)	Carl Roth GmbH & Co.KG, Karlsruhe
Blood Agar Basis Nr. 2	Oxoid Limited, Hampshire, UK
Horse blood, defibrinated	Oxoid Limited, Hampshire, UK
CAT-Supplement SR0174	Oxoid Limited, Hampshire, UK
Anerocult C	Merck KGaA, Darmstadt

Table B.6.: Restriction enzymes and DNA-marker

Enzyme or marker	Manufacturer
Lambda DNA *Eco*R I + *Hin*d III Marker (125 bp – 21226 bp)	Fermentas GmbH, St. Leon Rot
1 kb DNA-Ladder (250 bp – 10000 bp)	Fermentas GmbH, St. Leon Rot
pUC19-Marker (34 bp – 501 bp)	Fermentas GmbH, St. Leon Rot
Lysozym	Fermentas GmbH, St. Leon Rot
Proteinase K	Fermentas GmbH, St. Leon Rot
*Bsu*RI	Fermentas GmbH, St. Leon Rot
*Hin*6I	Fermentas GmbH, St. Leon Rot
*Rsa*I	Fermentas GmbH, St. Leon Rot
*Nco*I	Fermentas GmbH, St. Leon Rot
*Sal*I	Fermentas GmbH, St. Leon Rot
*Sca*I	Fermentas GmbH, St. Leon Rot
Taq Polymerase (native, ohne BSA) 1 U/µl	Fermentas GmbH, St. Leon Rot

Table B.8.: Primer used and designed in this study

Name	Sequence (5'-3')	Reference
BUTZ	CCT GGA CTT GAC ATA GTA AGA ATG A	Houf et al. (2000)
ARCO	CGT ATT CAC CGT AGC ATA GC	Houf et al. (2000)
SKIR	GGC GAT TTA CTG GAA CAC A	Houf et al. (2000)
CRY1	TGC TGG AGC GGA TAG AAG TA	Houf et al. (2000)
CRY2	AAC AAC CTA CGT CCT TCG AC	Houf et al. (2000)
TMBUTZFMM	AAA AAA TAC TTT CTT GGT CTT GTG GTG TA	Brightwell et al. (2007)
TMBUTZR	AAC AAC ACC TTT GTA TCT CAT TTT TTT G	Brightwell et al. (2007)
TMBUTZ	FAM-TTG GAC CAG TAA AAG ATT ATG AGT GTC TTT GTG GTA AA-BHQ1	Brightwell et al. (2007)
CRYFMM	AAG TGT AGA CGA TGG CAA ATT CG	Brightwell et al. (2007)
CRYRMM	CGA CCC ACT ATT CCA TCA GTG TG	Brightwell et al. (2007)
CRY	FAM-CCA ATA CCA ACA TAT AAG CGC GAT GTG GG-BHQ1	Brightwell et al. (2007)
16Sfor (27F)	AGA GTT TGA TCM TGG CTC AG	Lane (1991)
16Srev (1429R)	TAC GGY TAC CTT GTT ACG ACT T	Lane (1991)
Y1	TTA CCG GAC GCC GAG CTG TGG CGT	Darrasse et al. (1994)
Y2	CAG GAA GAT GTC GTT ATC GCG AGT	Darrasse et al. (1994)
pelB1	CAG CAC AAA CAG CAC CAG CG	Yap et al. (2004)
pelB2	GGG CCA CCG TTG TTG GTG CA	Yap et al. (2004)
G1	GAA GTC GTA ACA AGG	Toth et al. (2001)
L1	CAA GCA TCC ACC GT	Toth et al. (2001)
#188	ATG AAA TAC CTA YTG CCT	this study
#189	GCT GCT GTC AGY ACB GCM	this study
#194	CCG CTC ATC ATC ACC TAT	this study
#195	TGT GAT GGT AAG TCA GGT	this study
#238	ARA AGC TTA TGG TGC AA	this study
#239	CAA ACA ACA CCT TTG TAT CT	this study
#240	CGM ACG GGT GAG TAA TRT ATM G	this study
#241	TAY YTT AGC ATC CCC GCT	this study
#243	CAC CTT CCT CCT ACT TGC GT	this study
#295	TAA TAC GAC TCA CTA TAG GGA GAG TTT GAT CWT GGC TC	this study
#296	ATT TAG GTG ACA CTA TAG AAT ACG GYT ACC TTG TTA GGA CTT	this study
#346	CAT GCA GGC ATG AGT AGC GAT A	this study
#347	CCT GTG TCG GTT TAC GGT ACG	this study
#422	ACG GTA CGG GCA ACA TAT AA	this study
#431	CAT ATA AGC GCG ATG TGG	this study
#432	CAT ATA AGC GCG ATG TTG	this study
#433	ACG GTA CGG GCA ACA TAT AAT A	this study
#436	GAA GAT AAT GAC GGT ATT ATA TG	this study
#442	GTC CAC CAA ATA CTG TCC TTC TAG TCC ATC TCC TCC GAA GAG	this study
#443	CTA GAA GGA CAG TAT TTG GTG GAC CTA AGT TTA TTA TAT GTT GCC	this study
#448	FAM-TGG CTT TTC TTG GCA CGA CAG TAT CAT CGA TTC -BHQ1	this study
#449	JOE-CTA GAA GGA CAG TAT TTG GTG GAC-BHQ1	this study

Red color of base indicates a missmatch.

Table B.10.: Reference strains used in this study

Strain	Collection
Arcobacter nitrofigilis DSMZ 7299	DSMZ, Germany
Arcobacter skirrowii DSMZ 7302	DSMZ, Germany
Arcobacter butzleri DSMZ 8739	DSMZ, Germany
Arcobacter cibarius DSMZ 17680	DSMZ, Germany
Arcobacter cryaerophilus DSMZ 7289	DSMZ, Germany
Arcobacter halophilus DSMZ 18005	DSMZ, Germany
Arcobacter mytili LMG 24559	LMG, Belgium
Arcobacter thereius DSMZ 23385	DSMZ, Germany
Arcobacter trophiarum LMG 25534	LMG 25534T
Arcobacter marinus JCM 15502	JCM, Japan
Pectobacterium carotovorum ssp. *carotovorum* DSMZ 30168	DSMZ, Germany
Pectobacterium carotovorum ssp. *atrosepticum* DSMZ 18077	DSMZ, Germany
Pectobacterium carotovorum ssp. *betavascolarum* DSMZ 18076	DSMZ, Germany
Pectobacterium cypripedii DSMZ 3873	DSMZ, Germany
Pectobacterium wasabiae DSMZ 18074	DSMZ, Germany
Pseudomonas fluorescens DSMZ 50090	DSMZ, Germany
Dickeya chrysanthemi DSMZ 4610	DSMZ, Germany
Listeria innocua DSMZ 20649	DSMZ, Germany
Escherichia coli DSMZ 1116	DSMZ, Germany
Lactobacillus delbrueckii DSMZ 20074	DSMZ, Germany

C. Additional Tables and Figures

C.1. Detection of *Arcobacter* and *Pectobacterium* by PCR

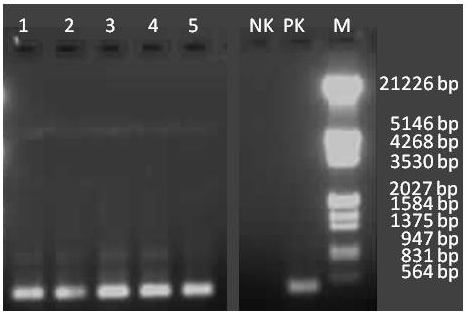

Figure C.1.: Determination of annealing temperature for primer pair #194/#195. 1 = 50°C; 2 = 52°C; 3 = 54°C; 4 = 56°C; 5 = 60°C; Template = gDNA of *P. carotovorum* ssp. *carotovorum*; NK = negative control; M = marker.

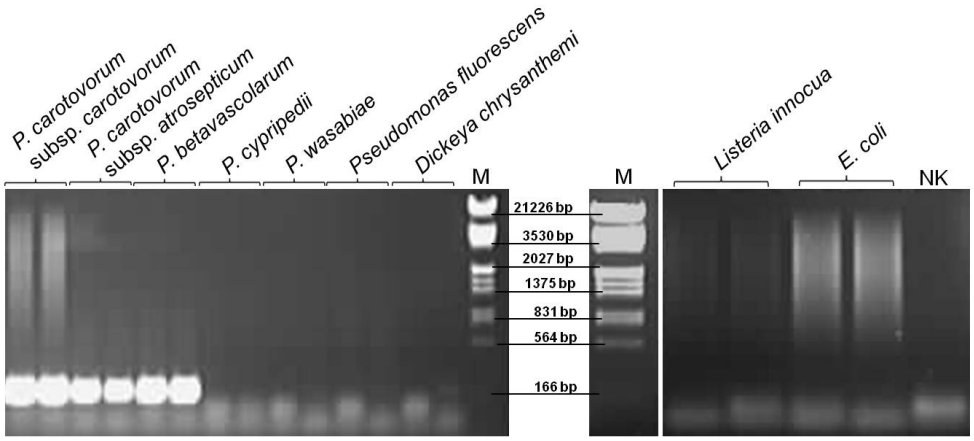

Figure C.2.: Specificity of qPCR-Assay targeting the *mdh*-gene of *Pectobacterium* spp. showing successful amplification of *P. carotovorum* ssp. *carotovorum*, *P. carotovorum* ssp. *atrosepticum*, *P. betavascolarum*. Template = gDNAs of different species; M = marker; NK = negative control.

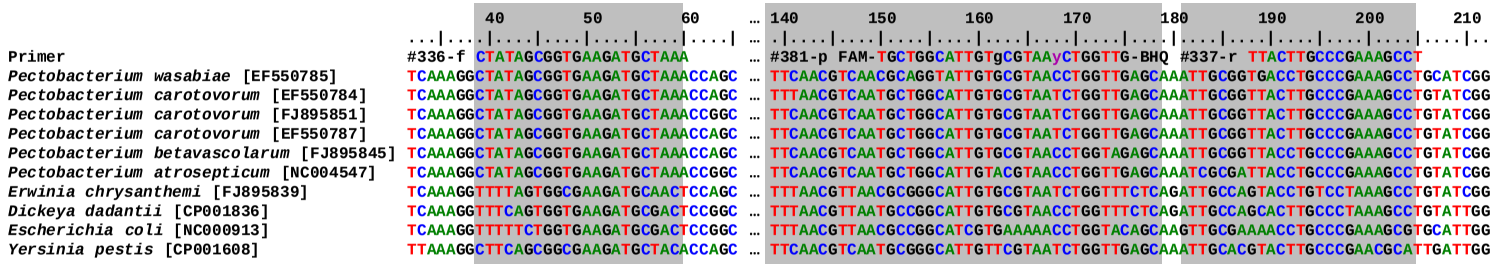

Figure C.3.: Alignment of *mdh*-genes of *Pectobacterium* spp. showing primer binding sites (areas marked in grey). Accession numbers are shown in brackets.

C.2. Genetic diversity of *Arcobacter* in a spinach-processing line

Table C.2.: Isolated clones with their designated OTU number and the nearest matching clone according to p-distance. Sequences diverging more than 1% were considered to belonging to different species. P-distances were calculated with the ARB-software using the Neighbor-Joining algorithm with Olson-correction.

OTU	Name	Affiliation according to BLAST search	Identity	Affiliation according to dendrogram
1	ATB-KS-13809	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
1	ATB-KS-13884	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
2	ATB-KS-13814	*Arcobacter defluvii* [HQ115596]	99%	*Arcobacter* sp.
2	ATB-KS-13860	*Arcobacter defluvii* [HQ115596]	99%	*Arcobacter* sp.
2	ATB-KS-14149	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
5	ATB-KS-13828	*Arcobacter defluvii* [HQ115596]	99%	*Arcobacter* sp.
5	ATB-KS-13875	*Arcobacter defluvii* [HQ115596]	97%	*Arcobacter* sp.
5	ATB-KS-14111	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
5	ATB-KS-17097	*Arcobacter defluvii* [HQ115596]	97%	*Arcobacter* sp.
6	ATB-KS-13816	*Arcobacter defluvii* [HQ115596]	97%	*Arcobacter* sp.
6	ATB-KS-13862	*Arcobacter defluvii* [HQ115596]	97%	*Arcobacter* sp.
6	ATB-KS-13887	*Arcobacter defluvii* [HQ115596]	96%	*Arcobacter* sp.
6	ATB-KS-16935	*Arcobacter defluvii* [HQ115596]	96%	*Arcobacter* sp.

OTU	Name	Affiliation according to BLAST search	Identity	Affiliation according to dendrogram
6	ATB-KS-17083	*Arcobacter defluvii* [HQ115596]	97%	*Arcobacter* sp.
7	ATB-KS-13832	*Arcobacter cryaerophilus* [FR682113]	99%	*A. cryaerophilus*
8	ATB-KS-13874	*Arcobacter cryaerophilus* [FR682113]	99%	*A. cryaerophilus*
8	ATB-KS-16271	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
10	ATB-LH-18668	*Arcobacter defluvii* [HQ115596]	97%	*Arcobacter* sp.
11	ATB-KS-13812	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
11	ATB-KS-13835	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
11	ATB-KS-13861	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
11	ATB-KS-13865	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
11	ATB-KS-13902	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
12	ATB-LH-17945	*Arcobacter defluvii* [HQ115596]	99%	*Arcobacter* sp.
19	ATB-KS-13898	*Arcobacter* sp. HME6665 [HM590830]	99%	*Arcobacter* sp.
21	ATB-KS-13844	*Arcobacter defluvii* [HQ115596]	99%	*Arcobacter* sp.
23	ATB-KS-13838	*Arcobacter defluvii* [HQ115596]	99%	*Arcobacter* sp.
24	ATB-KS-14115	*Arcobacter defluvii* [HQ115596]	99%	*Arcobacter* sp.
25	ATB-KS-14124	*Arcobacter* sp. HME6665 [HM590830]	99%	*A. butzleri*
26	ATB-KS-15827	*Arcobacter defluvii* [HQ115596]	99%	*Arcobacter* sp.

OTU	Name	Affiliation according to BLAST search	Identity	Affiliation according to dendrogram
28	ATB-KS-14134	*Arcobacter* sp. CpA_b6 [FN397894]	97%	*Arcobacter* sp.
29	ATB-KS-17072	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
30	ATB-KS-15932	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
31	ATB-KS-15954	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
33	ATB-KS-16064	*Arcobacter defluvii* [HQ115596]	99%	*Arcobacter* sp.
34	ATB-KS-14144	*Arcobacter* sp. CpA_b6 [FN397894]	97%	*Arcobacter* sp.
37	ATB-KS-16779	*Arcobacter defluvii* [HQ115596]	97%	*Arcobacter* sp.
38	ATB-KS-16249	*Arcobacter defluvii* [HQ115596]	97%	*Arcobacter* sp.
38	ATB-KS-16910	*Arcobacter defluvii* [HQ115596]	99%	*Arcobacter* sp.
39	ATB-KS-16909	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
40	ATB-KS-17044	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
43	ATB-KS-17089	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
44	ATB-KS-16844	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
45	ATB-KS-17132	*Arcobacter defluvii* [HQ115596]	97%	*Arcobacter* sp.
46	ATB-LH-18423	*Arcobacter defluvii* [HQ115596]	96%	*Arcobacter* sp.
47	ATB-LH-18434	*Arcobacter defluvii* [HQ115596]	99%	*Arcobacter* sp.
48	ATB-LH-18100	*Arcobacter nitrofigilis* [CP001999]	99%	*A. nitrofigilis*

OTU	Name	Affiliation according to BLAST search	Identity	Affiliation according to dendrogram
49	ATB-LH-18106	*Arcobacter nitrofigilis* [CP001999]	99%	*A. nitrofigilis*
50	ATB-LH-18157	*Arcobacter nitrofigilis* [CP001999]	99%	*A. nitrofigilis*
50	ATB-LH-18294	*Arcobacter nitrofigilis* [CP001999]	99%	*A. nitrofigilis*
51	ATB-LH-18143	*Arcobacter butzleri* [AP012047]	100%	*A. butzleri*
53	ATB-LH-18379	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
54	ATB-LH-18565	*Arcobacter nitrofigilis* [CP001999]	99%	*A. nitrofigilis*
55	ATB-LH-18179	*Arcobacter nitrofigilis* [CP001999]	99%	*A. nitrofigilis*
56	ATB-LH-18607	*Arcobacter nitrofigilis* [CP001999]	99%	*A. nitrofigilis*
57	ATB-LH-18765	*Arcobacter defluvii* [HQ115596]	99%	*A. defluvii*
58	ATB-KS-14093	*Arcobacter defluvii* [HQ115596]	96%	*Arcobacter* sp.
59	ATB-LH-18834	*Arcobacter nitrofigilis* [CP001999]	99%	*A. nitrofigilis*
60	ATB-LH-18662	*Arcobacter defluvii* [HQ115596]	96%	*Arcobacter* sp.
60	ATB-LH-18835	*Arcobacter defluvii* [HQ115596]	97%	*Arcobacter* sp.
61	ATB-KS-17054	*Arcobacter defluvii* [HQ115596]	97%	*Arcobacter* sp.
62	ATB-KS-16281	*Arcobacter cryaerophilus* [FR682113]	99%	*A. cryaerophilus*

Table C.1.: Chao-Jaccard indices for clone libraries of 2007 and 2009

Chao-Jaccard	II-S1	II-WW1	II-WW2	II-WW3	IV-BW	IV-S0	IV-S1	IV-S2	IV-S3	IV-S4	IV-TW	IV-WW1	IV-WW2	IV-WW3
II-S1														
II-WW1	0.805													
II-WW2	0.894	0.814												
II-WW3	0.831	0.791	0.876											
IV-BW	0.115	0.126	0.228	0.087										
IV-S0	0.01	0.011	0.031	0.086	0									
IV-S1	0.595	0.323	0.47	0.508	0.262	0.661								
IV-S2	0.899	0.63	0.895	0.635	0.319	0.385	0.713							
IV-S3	0.845	0.639	0.905	0.729	0.247	0.372	0.798	0.882						
IV-S4	0.607	0.209	0.477	0.274	0.492	0.299	0.947	0.687	0.811					
IV-TW	0.031	0.011	0.062	0.069	0.462	0.387	0.964	0.649	0.567	0.959				
IV-WW1	0.898	0.853	0.923	0.879	0.092	0.111	0.702	0.948	0.911	0.699	0.133			
IV-WW2	0.844	0.782	0.894	0.879	0.031	0.041	0.83	0.9	0.921	0.825	0.051	0.931		
IV-WW3	0.92	0.803	0.948	0.861	0.284	0.02	0.627	0.903	0.932	0.658	0.088	0.932	0.896	

C.3. Development of a multiplex-PCR detecting *Arcobacter* species

Table C.3.: List of primer pairs design for the *Arcobacter* multiplex-PCR like targeted species, target gene, amplicon size, primer ID (defined by the author) and sequence. These primers are referred to as set A.

Species	Target	Amplicon size	Primer	Sequence (5'-3')
A. butzleri LMG 10828	*rpo*B	150 bp	Abutz-F	AGGTGACACTATAGAATATGGAA AAATTATTGAACCATCG
			Abutz-R	GTACGACTCACTATAGGGAGCAT CATCATGTTCTAAGAATGGG
A. nitrofigilis LMG 7604	*rpo*B	255 bp	Anitro-F	AGGTGACACTATAGAATAGAGAT GTTCACGCAACTCACTAC
			Anitro-R	GTACGACTCACTATAGGGAGAGG CTCAACAATTTTACCATCG
A. skirrowii LMG 6621	*rpo*B	402 bp	Askir-F	AGGTGACACTATAGAATAAATCC ACTATCTGAAGTTACTCAC
			Askir-R	GTACGACTCACTATAGGGATCAA TTAAATCAACTTTATTTCTC
A. cryaerophilus LMG 7536	*rpo*B	290 bp	Acry-F	AGGTGACACTATAGAATAAGAAG CTTATGGTGCAACTG
			Acry-R	GTACGACTCACTATAGGGATAAT TTTAATTGGAAAGCAAC
A. halophilus DSM 18005	*gyr*A	53 bp	Ahalo-F	AGGTGACACTATAGAATACTCAA TGAGAGCTCCTTTAGTTGAC
			Ahalo-R	GTACGACTCACTATAGGGACATC AACTGAACCAAAGTTTCCC
A. cibarius LMG 21996	*gyr*A	308 bp	Aciba-F	AGGTGACACTATAGAATAAGTAC ACCGAAGAATCTTATATGCG
			Aciba-R	GTACGACTCACTATAGGGACAAA GTTTACAGTATCTTTATCTAAATCC
A. mytili LMG 24559	16S rRNA gene	434 bp	Amyt-F	AGGTGACACTATAGAATAAGTTG GAAACGACTGCTAATGT
			Amyt-R	GTACGACTCACTATAGGGAGATTT CACTCCTGACTTATCG
A. defluvii ATB-LH-6148	16S rRNA gene	274 bp	Asp-6148-F	AGGTGACACTATAGAATAACCTGC CCTCWAGAAAGGA
			Asp-16S-R	GTACGACTCACTATAGGGACGGCG TTGCTGCATCAGAC
Arcobacter sp. ATB-KS-14144	16S rRNA gene	222 bp	Asp-14144-F	AGGTGACACTATAGAATACGCCTT TTGAACGTAAGCTC
			Asp-16S-R	GTACGACTCACTATAGGGACGGC GTTGCTGCATCAGAC
			uni-multi-F	Cy5-AGGTGACACTATAGAATA
			uni-multi-R	GTACGACTCACTATAGGGA

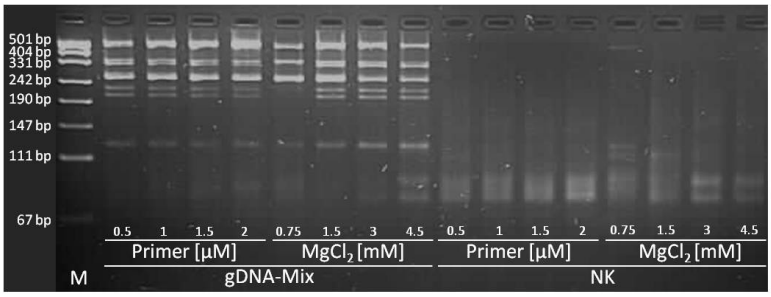

Figure C.4.: Optimization of primer- and $MgCl_2$- concentration. Template was a mix of all gDNAs. $MgCl_2$- concentration during primer optimization was 1.5 mM. Primer concentration during optimization of $MgCl_2$was 0.5 µM. M = marker; NK = negative control.

Table C.4.: List of primer properties for extended *Arcobacter* multiplex assay like targeted species, target gene, amplicon size, primer ID (defined by the author) and sequence. These primers are referred to as set B.

Species	Target	Amplicon size	Primer	Sequence (5'-3')
A. nitrofigilis DSMZ 7299T	23S rRNA gene	125 bp	493	AGGTGACACTATAGAATACG
				ATTCTTCGGAATCAAGTC
			494	GTACGACTCACTATAGGGAG
				GTTTACGGTACGGGCAACAA
A. thereius DSMZ 23385T	*rpo*B	209 bp	504	AGGTGACACTATAGAATATTC
				ACAGCAACTCAAGAAGAAGCG
			505	GTACGACTCACTATAGGGAACCC
				ATAACCATTTGTGAAGAGATATGA
A. halophilus DSMZ 18005T	*gyr*A	225 bp	497	AGGTGACACTATAGAATACTC
				AATGAGAGCTCCTTTAGTTGAC
			498	GTACGACTCACTATAGGGAAAT
				ACAGCAGGTTCTTTTAAAGTG
A. marinus JCM 15502T	*gyr*A	264 bp	499	AGGTGACACTATAGAATACAG
				TTTATGATGCATTGGTAAGAC
			498	GTACGACTCACTATAGGGAAAT
				ACAGCAGGTTCTTTTAAAGTG
A. defluvii ATB-LH-6148	*rpo*B	270 bp	506	AGGTGACACTATAGAATAGATT
				TATCGAAGCTCCATAAAACC
			505	GTACGACTCACTATAGGGAACCC
				ATAACCATTTGTGAAGAGATATGA
A. skirrowii DSMZ 7302T	*rpo*B	278 bp	507	AGGTGACACTATAGAATAGATTT
				AGGATTTATTGAAGCGCCATGC
			505	GTACGACTCACTATAGGGAACCC
				ATAACCATTTGTGAAGAGATATGA
A. cryaerophilus DSMZ 7289T	*rpo*B	327 bp	508	AGGTGACACTATAGAATAAG
				AAGCTTATGGTGCAACTG
			509	GTACGACTCACTATAGGGATA
				ATTTTAATTGGAAAGCAAC
A. butzleri DSMZ 8739T	*hsp*60	341 bp	502	AGGTGACACTATAGAATAAAA
				TCTTCAAAAGTTGTAGCAAGC
			503	GTACGACTCACTATAGGGAGG
				TCTTCCTGATTGATTTACTGA
A. cibarius DSMZ 17680T	*gyr*A	345 bp	500	AGGTGACACTATAGAATAAGT
				ACACCGAAGAATCTTATATGCG
			501	GTACGACTCACTATAGGGACAAAG
				TTTACAGTATCTTTATCTAAATCC
A. mytili LMG 24559T	16S rRNA gene	471 bp	491	AGGTGACACTATAGAATAAGT
				TGGAAACGACTGCTAATGT
			492	GTACGACTCACTATAGGGAGA
				TTTCACTCCTGACTTATCG
A. trophiarum LMG 25534T	23S rRNA gene	509 bp	495	AGGTGACACTATAGAATATGA
				ACTAATTGGAAAGCTAGAGGG
			496	GTACGACTCACTATAGGGACC
				CGAGACTTTTCAACGTCAAT

C.4. Detection of *Arcobacter* spp. by qPCR

Table C.5.: Cells detected with an *A. butzleri*-specific assay targeting the *rpo*-gene in spiked and unspiked samples of sampling IV-07/2009 and V-10/2009 (Brightwell et al., 2007). Samples designated with "x" were spiked with 10^9 cells of *A. butzleri*.

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
IV-TW			not analyzed		
IV-TWx 0.5 ng	23.42	5.73	1.12E+07		
IV-TWx 0.5 ng	23.37	5.73	1.16E+07		
IV-TWx 0.5 ng	23.48	5.73	1.08E+07	1.17E+07	1.75E+06
IV-TWx 1 ng	22.1	5.73	1.42E+07		
IV-TWx 1 ng	22.21	5.73	1.32E+07		
IV-TWx 1 ng	22.69	5.73	9.37E+06		
IV-WW1 1 ng	33.29	82.84	0.00E+00		
IV-WW1 1 ng	34.14	82.84	5.73E+03	3.98E+03	3.45E+03
IV-WW1 1 ng	34.03	82.84	6.21E+03		
IV-WW1x 0.5 ng	22.47	56.61	9.93E+07		
IV-WW1x 0.5 ng	22.91	56.61	7.22E+07		
IV-WW1x 0.5 ng	22.44	56.61	1.01E+08	9.26E+07	1.05E+07
IV-WW1x 1 ng	21.6	56.61	9.26E+07		
IV-WW1x 1 ng	21.54	56.61	9.61E+07		
IV-WW1x 1 ng	21.58	56.61	9.41E+07		
IV-WW2 1 ng	30.37	60.47	0.00E+00		
IV-WW2 1 ng	30.34	60.47	6.32E+04	4.09E+04	3.55E+04
IV-WW2 1 ng	30.43	60.47	5.95E+04		
IV-WW2x 0.5 ng	27.93	63.35	2.23E+06		
IV-WW2x 0.5 ng	28.65	63.35	1.34E+06		
IV-WW2x 0.5 ng	28.1	63.35	1.97E+06	1.75E+06	3.16E+05
IV-WW2x 1 ng	27.41	63.35	1.62E+06		
IV-WW2x 1 ng	27.45	63.35	1.58E+06		
IV-WW2x 1 ng	27.31	63.35	1.75E+06		
IV-WW3 1 ng	32.56	810.17	0.00E+00		
				1.46E+05	1.27E+05

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
IV-WW3 1 ng	32.25	810.17	2.17E+05		
IV-WW3 1 ng	32.22	810.17	2.22E+05		
IV-WW3x 0.5 ng	26.11	36.10	4.67E+06		
IV-WW3x 0.5 ng	26.12	36.10	4.64E+06		
IV-WW3x 0.5 ng	26.22	36.10	4.32E+06	5.00E+06	7.79E+05
IV-WW3x 1 ng	24.95	36.10	5.36E+06		
IV-WW3x 1 ng	25.17	36.10	4.59E+06		
IV-WW3x 1 ng	24.7	36.10	6.43E+06		
IV-BW 1 ng	29.07	22.63	5.86E+04		
IV-BW 1 ng	29.23	22.63	5.26E+04	3.71E+04	3.23E+04
IV-BW 1 ng	Undetermined	22.63	0.00E+00		
IV-BWx 0.5 ng	25.54	4.96	9.64E+05		
IV-BWx 0.5 ng	25.14	42.42	1.10E+07		
IV-BWx 0.5 ng	25.29	42.42	9.89E+06	8.53E+06	3.77E+06
IV-BWx 1 ng	24.23	42.42	1.06E+07		
IV-BWx 1 ng	24.42	42.42	9.21E+06		
IV-BWx 1 ng	24.37	42.42	9.57E+06		
IV-S01 ng	37.44	7.59	0.00E+00		
IV-S0 1 ng	38.11	7.59	3.07E+01	1.70E+01	1.56E+01
IV-S0 1 ng	38.7	7.59	2.01E+01		
IV-S0x 0.5 ng	20.43	4.40	3.33E+07		
IV-S0x 0.5 ng	21.14	4.40	2.00E+07		
IV-S0x 0.5 ng	20.97	4.40	2.25E+07	2.72E+07	5.67E+06
IV-S0x 1 ng	19.56	4.40	3.10E+07		
IV-S0x 1 ng	19.9	4.40	2.42E+07		
IV-S0x 1 ng	19.5	4.40	3.23E+07		
IV-S11 ng	37.85	57.34	0.00E+00		
IV-S1 1 ng	37.75	57.34	2.99E+02	1.88E+02	1.64E+02
IV-S1 1 ng	37.92	57.34	2.65E+02		
IV-S1x 0.5 ng	21.34	6.93	2.73E+07	2.46E+07	3.31E+06
IV-S1x 0.5 ng	21.24	6.93	2.93E+07		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
IV-S1x 0.5 ng	21.6	6.93	2.27E+07		
IV-S1x 1 ng	20.55	6.93	2.39E+07		
IV-S1x 1 ng	20.52	6.93	2.45E+07		
IV-S1x 1 ng	20.8	6.93	2.00E+07		
IV-S2			not analyzed		
IV-S2x			not analyzed		
IV-S3 1 ng	33.28	7.54	0.00E+00		
IV-S3 1 ng	35.05	7.54	2.71E+02	2.24E+02	2.05E+02
IV-S3 1 ng	34.51	7.54	4.01E+02		
IV-S3x 0.5 ng	22.19	6.92	1.48E+07		
IV-S3x 0.5 ng	22.24	6.92	1.43E+07		
IV-S3x 0.5 ng	22.36	6.92	1.31E+07	1.43E+07	1.40E+06
IV-S3x 1 ng	21.34	6.92	1.36E+07		
IV-S3x 1 ng	21.38	6.92	1.32E+07		
IV-S3x 1 ng	21.04	6.92	1.69E+07		
IV-S4			not analyzed		
IV-S4x			not analyzed		
V-TW			not analyzed		
V-TWx 0.5 ng	28.97	1.01	3.96E+04		
V-TWx 0.5 ng	27.51	1.01	1.11E+05		
V-TWx 0.5 ng	27.6	1.01	1.04E+05	8.49E+04	3.95E+04
V-TWx 1 ng	Undetermined	1.01	0.00E+00		
V-TWx 1 ng	Undetermined	1.01	0.00E+00		
V-TWx 1 ng	Undetermined	1.01	0.00E+00		
V-WW1 1 ng	Undetermined	135.42	0.00E+00		
V-WW1 1 ng	37.29	135.42	2.51E+03	2.00E+03	1.79E+03
V-WW1 1 ng	36.82	135.42	3.48E+03		
V-WW1x 0.5 ng	26.04	15.87	4.91E+06		
V-WW1x 0.5 ng	25.89	15.87	5.46E+06		
V-WW1x 0.5 ng	26.53	15.87	3.48E+06	2.86E+06	2.04E+06

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
V-WW1x 1 ng	27.36	15.87	9.70E+05		
V-WW1x 1 ng	27.38	15.87	9.51E+05		
V-WW1x 1 ng	26.84	15.87	1.39E+06		
V-WW2 1 ng	Undetermined	163.77	0.00E+00		
V-WW2 1 ng	38.86	163.77	1.00E+03	7.38E+02	6.48E+02
V-WW2 1 ng	38.59	163.77	1.21E+03		
V-WW2x 0.5 ng	24.57	12.52	1.09E+07		
V-WW2x 0.5 ng	24.33	12.52	1.30E+07		
V-WW2x 0.5 ng	24.41	12.52	1.22E+07	7.42E+06	5.12E+06
V-WW2x 1 ng	25.47	12.52	2.90E+06		
V-WW2x 1 ng	25.89	12.52	2.15E+06		
V-WW2x 1 ng	25.26	12.52	3.35E+06		
V-WW3 1 ng	37.2	74.42	1.47E+03		
V-WW3 1 ng	37.19	74.42	1.48E+03	1.33E+03	2.49E+02
V-WW3 1 ng	37.68	74.42	1.04E+03		
V-WW3x 0.5 ng	24.28	16.26	1.74E+07		
V-WW3x 0.5 ng	24.61	16.26	1.38E+07		
V-WW3x 0.5 ng	24.8	16.26	1.21E+07	1.26E+07	2.66E+06
V-WW3x 1 ng	24.1	16.26	9.86E+06		
V-WW3x 1 ng	23.86	16.26	1.17E+07		
V-WW3x 1 ng	23.95	16.26	1.10E+07		
V-BW 1 ng	30.63	44.03	8.89E+04		
V-BW 1 ng	32.25	44.03	2.84E+04	8.22E+04	5.08E+04
V-BW 1 ng	30.1	44.03	1.29E+05		
V-BWx 0.5 ng	Undetermined	4.20	0.00E+00		
V-BWx 0.5 ng	39.96	4.20	7.11E+01		
V-BWx 0.5 ng	Undetermined	4.20	0.00E+00	1.04E+02	1.00E+02
V-BWx1 ng	38.23	4.20	1.20E+02		
V-BWx 1 ng	37.22	4.20	2.46E+02		
V-BWx 1 ng	37.6	4.20	1.87E+02		
V-S0			not analyzed		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
V-S0x			not analyzed		
V-S1			not analyzed		
V-S1x 0.5 ng	21.44	1.37	1.08E+07	9.70E+06	1.20E+06
V-S1x 0.5 ng	21.92	1.37	7.70E+06		
V-S1x 0.5 ng	21.49	1.37	1.04E+07		
V-S1x 1 ng	20.49	1.37	1.06E+07		
V-S1x 1 ng	20.73	1.37	8.88E+06		
V-S1x 1 ng	20.6	1.37	9.83E+06		
V-S2			not analyzed		
V-S2x 0.5 ng	34.45	0.59	4.83E+02	9.13E+02	4.48E+02
V-S2x 0.5 ng	34.05	0.59	6.42E+02		
V-S2x 0.5 ng	34.24	0.59	5.60E+02		
V-S2x 1 ng	32.35	0.59	1.06E+03		
V-S2x 1 ng	31.71	0.59	1.67E+03		
V-S2x 1 ng	32.35	0.59	1.06E+03		
V-S3			not analyzed		
V-S3x			not analyzed		
V-S4x 0.5 ng	26.34	94.54	2.36E+07	2.87E+07	5.07E+06
V-S4x 0.5 ng	25.99	94.54	3.03E+07		
V-S4x 0.5 ng	26.04	94.54	2.92E+07		
V-S4x 1 ng	24.69	94.54	3.78E+07		
V-S4x 1 ng	25.24	94.54	2.57E+07		
V-S4x 1 ng	25.23	94.54	2.58E+07		

Table C.6.: Cells detected with an *A. cryaerophilus*-specific assay in spiked and unspiked samples of sampling VI-06/2010 and VII-06/2010 (Brightwell et al., 2007). Samples designated with "x" were spiked with 10^8 cells of *A. cryaerophilus*.

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-TW 0.5 ng	35.12	3.92E-01	3.60E+02		
VI-TW 0.5 ng	35.27	3.92E-01	3.22E+02	3.06E+02	6.33E+01
VI-TW 0.5 ng	35.68	3.92E-01	2.36E+02		
VI-TWx 0.5 ng	22.97	2.34E+01	2.12E+08		
VI-TWx 0.5 ng	23.15	2.34E+01	1.84E+08		
VI-TWx 0.5 ng	22.8	2.34E+01	2.41E+08		
VI-TWx 1 ng	19.84	2.34E+01	2.36E+09		
VI-TWx 1 ng	21.74	2.34E+01	2.69E+08	5.61E+08	7.07E+08
VI-TWx 1 ng	21.89	2.34E+01	2.40E+08		
VI-TWx 10 ng	17.14	2.34E+01	8.75E+08		
VI-TWx 10 ng	18.34	2.34E+01	3.52E+08		
VI-TWx 10 ng	18.5	2.34E+01	3.13E+08		
VI-WW1 1 ng	37.77	5.45E+01	3.37E+03		
VI-WW1 1 ng	36.72	5.45E+01	7.44E+03	5.40E+03	2.88E+03
VI-WW1 1 ng	Undetermined	5.45E+01	0.00E+00		
VI-WW1x 0.5 ng	22.09	1.72E+01	3.02E+08		
VI-WW1x 0.5 ng	22.15	1.72E+01	2.88E+08		
VI-WW1x 0.5 ng	22.34	1.72E+01	2.50E+08		
VI-WW1x 1 ng	20.72	1.72E+01	4.25E+08		
VI-WW1x 1 ng	21.37	1.72E+01	2.61E+08	2.64E+08	7.52E+07
VI-WW1x 1 ng	21.35	1.72E+01	2.65E+08		
VI-WW1x 10 ng	18.5	1.72E+01	2.30E+08		
VI-WW1x 10 ng	18.67	1.72E+01	2.01E+08		
VI-WW1x 10 ng	19.02	1.72E+01	1.55E+08		
VI-WW2 1 ng	35.68	1.50E+02	4.50E+04		
VI-WW2 1 ng	35.41	1.50E+02	5.53E+04	5.18E+04	5.82E+03
VI-WW2 1 ng	35.42	1.50E+02	5.49E+04		
VI-WW2x 0.5 ng	24.12	3.34E+01	1.27E+08		
				1.55E+08	7.06E+07

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-WW2x 0.5 ng	24.02	3.34E+01	1.37E+08		
VI-WW2x 0.5 ng	24.19	3.34E+01	1.21E+08		
VI-WW2x 1 ng	Undetermined	3.34E+01	0.00E+00		
VI-WW2x 1 ng	22.59	3.34E+01	2.02E+08		
VI-WW2x 1 ng	22.76	3.34E+01	1.77E+08		
VI-WW2x 10 ng	19.36	3.34E+01	2.32E+08		
VI-WW2x 10 ng	19.42	3.34E+01	2.22E+08		
VI-WW2x 10 ng	19.74	3.34E+01	1.75E+08		
VI-WW3 1 ng	35.32	1.29E+02	5.13E+04		
VI-WW3 1 ng	35.09	1.29E+02	6.08E+04	5.99E+04	8.25E+03
VI-WW3 1 ng	34.95	1.29E+02	6.77E+04		
VI-WW3x 0.5 ng	24.42	2.83E+01	8.54E+07		
VI-WW3x 0.5 ng	24.81	2.83E+01	6.36E+07		
VI-WW3x 0.5 ng	25.16	2.83E+01	4.87E+07	7.98E+07	6.28E+07
VI-WW3x 1 ng	22.75	2.83E+01	1.51E+08		
VI-WW3x 1 ng	22.84	2.83E+01	1.41E+08		
VI-WW3x 1 ng	22.77	2.83E+01	1.48E+08		
VI-WW3x 10 ng	19.21	removed			
VI-WW3x 10 ng	38.16	from			
VI-WW3x 10 ng	38.57	analysis			
VI-BW 1 ng	37.45	8.92E+01	7.05E+03		
VI-BW 1 ng	37.37	8.92E+01	7.44E+03	7.76E+03	9.07E+02
VI-BW 1 ng	37.16	8.92E+01	8.78E+03		
VI-BWx 0.5 ng	23.48	1.83E+01	1.13E+08		
VI-BWx 0.5 ng	23.57	1.83E+01	1.05E+08		
VI-BWx 0.5 ng	24	1.83E+01	7.59E+07		
VI-BWx 1 ng	22.58	1.83E+01	1.11E+08		
VI-BWx 1 ng	22.6	1.83E+01	1.10E+08	9.44E+07	2.10E+07
VI-BWx 1 ng	22.6	1.83E+01	1.10E+08		
VI-BWx 10 ng	19.7	1.83E+01	9.86E+07		
VI-BWx 10 ng	20.27	1.83E+01	6.39E+07		
VI-BWx 10 ng	20.3	1.83E+01	6.26E+07		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-S0 1 ng	39.37	2.09E+01	2.87E+02		
VI-S0 1 ng	39.19	2.09E+01	3.29E+02	4.72E+02	2.85E+02
VI-S0 1 ng	38.02	2.09E+01	8.00E+02		
VI-S0x 0.5 ng	23.15	2.31E+01	1.40E+08		
VI-S0x 0.5 ng	23.61	2.31E+01	9.87E+07		
VI-S0x 0.5 ng	23.61	2.31E+01	9.90E+07		
VI-S0x 1 ng	21.81	2.31E+01	1.93E+08		
VI-S0x 1 ng	21.77	2.31E+01	2.00E+08	1.67E+08	5.35E+07
VI-S0x 1 ng	22.01	2.31E+01	1.67E+08		
VI-S0x 10 ng	18.4	2.31E+01	2.57E+08		
VI-S0x 10 ng	18.88	2.31E+01	1.79E+08		
VI-S0x 10 ng	Undetermined	2.31E+01	0.00E+00		
VI-S1 1 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1 1 ng	39.44	3.17E+01	0.00E+00	0.00E+00	0.00E+00
VI-S1 1 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1x 0.5 ng	22.27	2.43E+01	2.86E+08		
VI-S1x 0.5 ng	22.83	2.43E+01	1.86E+08		
VI-S1x 0.5 ng	23.29	2.43E+01	1.32E+08		
VI-S1x 1 ng	20.68	2.43E+01	4.79E+08		
VI-S1x 1 ng	21.15	2.43E+01	3.33E+08	2.65E+08	1.16E+08
VI-S1x 1 ng	20.96	2.43E+01	3.87E+08		
VI-S1x 10 ng	18.71	2.43E+01	2.13E+08		
VI-S1x 10 ng	18.72	2.43E+01	2.12E+08		
VI-S1x 10 ng	19.13	2.43E+01	1.55E+08		
VI-S2 1 ng	22.33	1.97E+01	1.11E+08		
VI-S2 1 ng	22.37	1.97E+01	1.07E+08	1.10E+08	2.60E+06
VI-S2 1 ng	22.32	1.97E+01	1.12E+08		
VI-S2x 0.5 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 0.5 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 0.5 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 1 ng	39.59	2.62E+01	0.00E+00		
				2.32E+02	5.77E+01

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-S2x 1 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 1 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 10 ng	37.04	2.62E+01	2.10E+02		
VI-S2x 10 ng	36.58	2.62E+01	2.97E+02		
VI-S2x 10 ng	37.19	2.62E+01	1.88E+02		
VI-S3 1 ng	38.17	3.27E+01	1.11E+03		
VI-S3 1 ng	38.1	3.27E+01	1.17E+03	9.92E+02	2.63E+02
VI-S3 1 ng	38.8	3.27E+01	6.91E+02		
VI-S3x 0.5 ng	21.15	7.48E+00	2.06E+08		
VI-S3x 0.5 ng	21.22	7.48E+00	1.97E+08		
VI-S3x 0.5 ng	21.16	7.48E+00	2.06E+08		
VI-S3x 1 ng	20.58	7.48E+00	1.59E+08		
VI-S3x 1 ng	20.62	7.48E+00	1.54E+08	1.46E+08	5.58E+07
VI-S3x 1 ng	20.6	7.48E+00	1.57E+08		
VI-S3x 10 ng	18.22	7.48E+00	9.54E+07		
VI-S3x 10 ng	18.57	7.48E+00	7.33E+07		
VI-S3x 10 ng	18.77	7.48E+00	6.28E+07		
VI-S4 1 ng	38.25	8.10E+01	2.59E+03		
VI-S4 1 ng	38.68	8.10E+01	1.87E+03	2.25E+03	3.59E+02
VI-S4 1 ng	38.41	8.10E+01	2.30E+03		
VI-S4x 0.5 ng	22.53	1.59E+01	1.54E+08		
VI-S4x 0.5 ng	23.04	1.59E+01	1.05E+08		
VI-S4x 0.5 ng	27.3	1.59E+01	4.40E+06		
VI-S4x 1 ng	21.17	1.59E+01	2.16E+08		
VI-S4x 1 ng	21.07	1.59E+01	2.34E+08	1.52E+08	8.82E+07
VI-S4x 1 ng	20.71	1.59E+01	3.06E+08		
VI-S4x 10 ng	18.9	1.59E+01	1.22E+08		
VI-S4x 10 ng	18.94	1.59E+01	1.18E+08		
VI-S4x 10 ng	18.99	1.59E+01	1.13E+08		
VII-BF1 1 ng	37.95	3.60E+02	1.96E+03		
VII-BF1 1 ng	37.63	3.60E+02	2.54E+03	2.43E+03	4.24E+02

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VII-BF1 1 ng	37.52	3.60E+02	2.79E+03		
VII-BF1x 0.5 ng	25.3	5.01E+01	1.96E+07		
VII-BF1x 0.5 ng	25.15	5.01E+01	2.21E+07		
VII-BF1x 0.5 ng	25.2	5.01E+01	2.13E+07		
VII-BF1x 1 ng	24.34	5.01E+01	2.16E+07		
VII-BF1x 1 ng	24.26	5.01E+01	2.32E+07	2.51E+07	5.55E+06
VII-BF1x 1 ng	24.35	5.01E+01	2.15E+07		
VII-BF1x 10 ng	20.98	5.01E+01	3.51E+07		
VII-BF1x 10 ng	21.12	5.01E+01	3.13E+07		
VII-BF1x 10 ng	21.17	5.01E+01	3.01E+07		
VII-BF2 1 ng	37.78	1.30E+02	8.12E+02		
VII-BF2 1 ng	37.46	1.30E+02	1.06E+03	1.01E+03	1.77E+02
VII-BF2 1 ng	37.35	1.30E+02	1.16E+03		
VII-BF2x 0.5 ng	25.35	1.72E+01	6.44E+06		
VII-BF2x 0.5 ng	25	1.72E+01	8.58E+06		
VII-BF2x 0.5 ng	25.17	1.72E+01	7.49E+06		
VII-BF2x 1 ng	23.62	1.72E+01	1.35E+07		
VII-BF2x 1 ng	23.81	1.72E+01	1.16E+07	6.83E+06	5.16E+06
VII-BF2x 1 ng	23.76	1.72E+01	1.20E+07		
VII-BF2x 10 ng	25.21	1.72E+01	3.62E+05		
VII-BF2x 10 ng	24.91	1.72E+01	4.63E+05		
VII-BF2x 10 ng	23.89	1.72E+01	1.08E+06		
VII-WW1 1 ng	32.69	1.04E+01	4.42E+03		
VII-WW1 1 ng	32.02	1.04E+01	7.71E+03	6.95E+03	2.25E+03
VII-WW1 1 ng	31.87	1.04E+01	8.72E+03		
VII-WW1x 0.5 ng	22.35	1.59E+01	7.14E+07		
VII-WW1x 0.5 ng	22.25	1.59E+01	7.73E+07		
VII-WW1x 0.5 ng	22.47	1.59E+01	6.45E+07		
VII-WW1x 1 ng	21.53	1.59E+01	7.03E+07		
VII-WW1x 1 ng	21.32	1.59E+01	8.43E+07	7.16E+07	1.84E+07
VII-WW1x 1 ng	21.91	1.59E+01	5.14E+07		
VII-WW1x 10 ng	18.41	1.59E+01	9.39E+07		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VII-WW1x 10 ng	18.42	1.59E+01	9.31E+07		
VII-WW1x 10 ng	19.48	1.59E+01	3.84E+07		
VII-WW2 1 ng	35.63	9.69E+00	3.61E+02		
VII-WW2 1 ng	35.53	9.69E+00	3.90E+02	4.28E+02	9.15E+01
VII-WW2 1 ng	35.16	9.69E+00	5.32E+02		
VII-WW2x 0.5 ng	22.45	1.40E+01	5.80E+07		
VII-WW2x 0.5 ng	22.21	1.40E+01	7.09E+07		
VII-WW2x 0.5 ng	22.74	1.40E+01	4.57E+07		
VII-WW2x 1 ng	21.51	1.40E+01	6.31E+07		
VII-WW2x 1 ng	21.16	1.40E+01	8.41E+07	5.50E+07	2.44E+07
VII-WW2x 1 ng	21.1	1.40E+01	8.90E+07		
VII-WW2x 10 ng	19.84	1.40E+01	2.51E+07		
VII-WW2x 10 ng	19.36	1.40E+01	3.74E+07		
VII-WW2x 10 ng	20.04	1.40E+01	2.13E+07		
VII-WW3 1 ng	Undetermined	2.57E+00	0.00E+00		
VII-WW3 1 ng	Undetermined	2.57E+00	0.00E+00	0.00E+00	0.00E+00
VII-WW3 1 ng	Undetermined	2.57E+00	0.00E+00		
VII-WW3x 0.5 ng	22.4	2.62E+01	1.12E+08		
VII-WW3x 0.5 ng	22.51	2.62E+01	1.03E+08		
VII-WW3x 0.5 ng	22.47	2.62E+01	1.07E+08		
VII-WW3x 1 ng	21.6	2.62E+01	1.09E+08		
VII-WW3x 1 ng	21.36	2.62E+01	1.34E+08	3.77E+08	3.11E+08
VII-WW3x 1 ng	21.27	2.62E+01	1.44E+08		
VII-WW3x 10 ng	24.12	2.62E+01	1.36E+06		
VII-WW3x 10 ng	24.75	2.62E+01	8.04E+05		
VII-WW3x 10 ng	22.76	2.62E+01	2.18E+07		

Table C.7.: Cells detected with *Arcobacter*-specific qPCR-Assay targeting the 16S rDNA gene in spiked and unspiked samples of sampling VIa VI VII. Samples designated with "x" were spiked with 10^8 cells of *A. cryaerophilus*.

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VIa-WW2 0.5 ng	26.67	7.15	1.15E+04		
VIa-WW2 0.5 ng	26.78	7.15	1.06E+04		
VIa-WW2 0.5 ng	26.99	7.15	9.19E+03	6.19E+03	4.72E+03
VIa-WW2 1 ng	29.17	7.15	1.04E+03		
VIa-WW2 1 ng	28.38	7.15	1.79E+03		
VIa-WW2 1 ng	27.57	7.15	3.09E+03		
VIa-WW2x 0.5 ng	13.98	8.98	2.36E+08		
VIa-WW2x 0.5 ng	13.91	8.98	2.48E+08		
VIa-WW2x 0.5 ng	14.16	8.98	2.10E+08	1.31E+08	1.12E+08
VIa-WW2x 1 ng	17.64	8.98	9.89E+06		
VIa-WW2x 1 ng	16.44	8.98	2.24E+07		
VIa-WW2x 1 ng	15.04	8.98	5.77E+07		
VIa-WW3 0.5 ng	30.14	6.10	9.28E+02		
VIa-WW3 0.5 ng	30.28	6.10	8.42E+02		
VIa-WW3 0.5 ng	29.78	6.10	1.18E+03	5.90E+02	4.46E+02
VIa-WW3 1 ng	31.59	6.10	1.73E+02		
VIa-WW3 1 ng	31.37	6.10	2.01E+02		
VIa-WW3 1 ng	31.28	6.10	2.13E+02		
VIa-WW3x 1 ng	14.04	9.26	1.17E+08		
VIa-WW3 1 ng	14.06	9.26	1.15E+08		
VIa-WW3 1 ng	14.41	9.26	9.14E+07	1.22E+08	2.91E+07
VIa-WW3 10 ng	14.17	9.26	1.08E+08		
VIa-WW3 10 ng	13.97	9.26	1.23E+08		
VIa-WW3 10 ng	13.43	9.26	1.77E+08		
VI-S0 0.5 ng	Undetermined	2.09E+01	0.00E+00		
VI-S0 0.5 ng	Undetermined	2.09E+01	0.00E+00		
VI-S0 0.5 ng	Undetermined	2.09E+01	0.00E+00	0.00E+00	0.00E+00
VI-S0 1 ng	Undetermined	2.09E+01	0.00E+00		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-S0 1 ng	Undetermined	2.09E+01	0.00E+00		
VI-S0 1 ng	Undetermined	2.09E+01	0.00E+00		
VI-S0x 0.5 ng	20.85	2.31E+01	1.06E+09		
VI-S0x 0.5 ng	22.86	2.31E+01	4.22E+08		
VI-S0x 0.5 ng	24.07	2.31E+01	2.42E+08		
VI-S0x 1 ng	22.54	2.31E+01	2.45E+08		
VI-S0x 1 ng	21.51	2.31E+01	3.93E+08	1.71E+08	1.42E+08
VI-S0x 1 ng	22.65	2.31E+01	2.32E+08		
VI-S0x 10 ng	20.09	2.31E+01	7.52E+07		
VI-S0x 10 ng	21.1	2.31E+01	4.73E+07		
VI-S0x 10 ng	21.74	2.31E+01	3.53E+07		
VI-S1 0.5 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1 0.5 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1 0.5 ng	Undetermined	3.17E+01	0.00E+00	0.00E+00	0.00E+00
VI-S1 1 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1 1 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1 1 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1x 0.5 ng	21.36	2.43E+01	8.81E+08		
VI-S1x 0.5 ng	21.85	2.43E+01	7.02E+08		
VI-S1x 0.5 ng	18.53				
VI-S1x 1 ng	20.43	2.43E+01	6.73E+08		
VI-S1x 1 ng	18.81	2.43E+01	1.42E+09	6.74E+08	4.95E+08
VI-S1x 1 ng	19.7	2.43E+01	9.46E+08		
VI-S1x 10 ng	21.02	2.43E+01	5.15E+07		
VI-S1x 10 ng	33.19				
VI-S1x 10 ng	21.46	2.43E+01	4.22E+07		
VI-S2 0.5 ng	19.35				
VI-S2 0.5 ng	35.78	1.97E+01	3.15E+05		
VI-S2 0.5 ng	35.17	1.97E+01	4.17E+05	4.55E+07	4.14E+07
VI-S2 1 ng	22.35	1.97E+01	7.55E+07		
VI-S2 1 ng	22.52	1.97E+01	6.98E+07		
VI-S2 1 ng	22.19	1.97E+01	8.15E+07		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-S2x 0.5 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 0.5 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 0.5 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 1 ng	Undetermined	2.62E+01	0.00E+00	0.00E+00	0.00E+00
VI-S2x 1 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 1 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 10 ng	Undetermined	2.62E+01	0.00E+00		
VI-S3 0.5 ng	Undetermined	3.27E+01	0.00E+00		
VI-S3 0.5 ng	Undetermined	3.27E+01	0.00E+00		
VI-S3 0.5 ng	Undetermined	3.27E+01	0.00E+00	0.00E+00	0.00E+00
VI-S3 1 ng	Undetermined	3.27E+01	0.00E+00		
VI-S3 1 ng	Undetermined	3.27E+01	0.00E+00		
VI-S3 1 ng	Undetermined	3.27E+01	0.00E+00		
VI-S3x 0.5 ng	23.36	7.48E+00	1.15E+08		
VI-S3x 0.5 ng	20.44	7.48E+00	4.45E+08		
VI-S3x 0.5 ng	21.38	7.48E+00	2.86E+08	2.52E+08	3.92E+07
VI-S3x 1 ng	19.91	7.48E+00	2.82E+08		
VI-S3x 1 ng	20.05	7.48E+00	2.66E+08		
VI-S3x 1 ng	20.59	7.48E+00	2.08E+08		
VI-S4 0.5 ng	Undetermined	8.10E+01	0.00E+00		
VI-S4 0.5 ng	Undetermined	8.10E+01	0.00E+00		
VI-S4 0.5 ng	Undetermined	8.10E+01	0.00E+00	0.00E+00	0.00E+00
VI-S4 1 ng	Undetermined	8.10E+01	0.00E+00		
VI-S4 1 ng	Undetermined	8.10E+01	0.00E+00		
VI-S4 1 ng	Undetermined	8.10E+01	0.00E+00		
VI-S4x 0.5 ng	21.59	1.59E+01	5.54E+08		
VI-S4x 0.5 ng	21.44	1.59E+01	5.93E+08		
VI-S4x 0.5 ng	21.44	1.59E+01	5.93E+08		
VI-S4x 1 ng	21.04	1.59E+01	3.56E+08		
VI-S4x 1 ng	21.12	1.59E+01	3.45E+08	3.73E+08	1.99E+08
VI-S4x 1 ng	21.21	1.59E+01	3.30E+08		
VI-S4x 10 ng	28.39				

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-S4x 10 ng	19.41	1.59E+01	7.60E+07		
VI-S4x 10 ng	18.16	1.59E+01	1.36E+08		
VI-TW 0.4 ng	Undetermined				
VI-TW 0.4 ng	Undetermined			0.00E+00	0.00E+00
VI-TW 0.4 ng	Undetermined				
VI-TWx 0.5 ng	20.49	2.34E+01	1.06E+09		
VI-TWx 0.5 ng	20.94	2.34E+01	8.53E+08		
VI-TWx 0.5 ng	20.9	2.34E+01	8.69E+08		
VI-TWx 1 ng	20.49	2.34E+01	5.29E+08		
VI-TWx 1 ng	20.29	2.34E+01	5.81E+08	5.39E+08	3.87E+08
VI-TWx 1 ng	21.06	2.34E+01	4.03E+08		
VI-TWx 10 ng	23.85	2.34E+01	1.06E+07		
VI-TWx 10 ng	19.23				
VI-TWx 10 ng	23.32	2.34E+01	1.37E+07		
VI-WW1 0.5 ng	Undetermined	5.45E+01	0.00E+00		
VI-WW1 0.5 ng	Undetermined	5.45E+01	0.00E+00		
VI-WW1 0.5 ng	Undetermined	5.45E+01	0.00E+00	0.00E+00	0.00E+00
VI-WW1 1 ng	Undetermined	5.45E+01	0.00E+00		
VI-WW1 1 ng	Undetermined	5.45E+01	0.00E+00		
VI-WW1 1 ng	Undetermined	5.45E+01	0.00E+00		
VI-WW1x 0.5 ng	20.88	1.72E+01	6.45E+08		
VI-WW1x 0.5 ng	21.72	1.72E+01	4.31E+08		
VI-WW1x 0.5 ng	23.75	1.72E+01	1.63E+08		
VI-WW1x 1 ng	21.4	1.72E+01	2.50E+08		
VI-WW1x 1 ng	20.13	1.72E+01	4.59E+08	3.07E+08	1.33E+08
VI-WW1x 1 ng	21.74	1.72E+01	2.13E+08		
VI-WW1x 10 ng	22.17	1.72E+01	1.74E+07		
VI-WW1x 10 ng	31.57	1.72E+01	1.94E+05		
VI-WW1x 10 ng	Undetermined	1.72E+01	0.00E+00		
VI-WW2 0.5 ng	Undetermined	1.50E+02	0.00E+00		
VI-WW2 0.5 ng	Undetermined	1.50E+02	0.00E+00	0.00E+00	0.00E+00

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-WW2 0.5 ng	Undetermined	1.50E+02	0.00E+00		
VI-WW2 1 ng	36.28	1.50E+02	5.95E+05		
VI-WW2 1 ng	37.66	1.50E+02	3.08E+05	2.43E+05	2.84E+05
VI-WW2 1 ng	36.43	1.50E+02	5.55E+05		
VI-WW2x 0.5 ng	22.46	3.34E+01	5.90E+08		
VI-WW2x 0.5 ng	23.07	3.34E+01	4.40E+08		
VI-WW2x 0.5 ng	22.61	3.34E+01	5.47E+08		
VI-WW2x 1 ng	21.53	3.34E+01	4.59E+08		
VI-WW2x 1 ng	21.48	3.34E+01	4.69E+08	4.00E+08	1.45E+08
VI-WW2x 1 ng	21.6	3.34E+01	4.43E+08		
VI-WW2x 10 ng	18.4	3.34E+01	2.05E+08		
VI-WW2x 10 ng	18.24	3.34E+01	2.21E+08		
VI-WW2x 10 ng	18.19	3.34E+01	2.26E+08		
VI-WW3 0.5 ng	33.38	1.29E+02	4.10E+06		
VI-WW3 0.5 ng	33.41	1.29E+02	4.04E+06		
VI-WW3 0.5 ng	33.02	1.29E+02	4.88E+06	2.79E+06	1.73E+06
VI-WW3 1 ng	34.95	1.29E+02	9.69E+05		
VI-WW3 1 ng	34.28	1.29E+02	1.34E+06		
VI-WW3 1 ng	34.16	1.29E+02	1.41E+06		
VI-WW3x 0.5 ng	21.82	2.83E+01	6.77E+08		
VI-WW3x 0.5 ng	22.28	2.83E+01	5.42E+08		
VI-WW3x 0.5 ng	22.18	2.83E+01	5.68E+08		
VI-WW3x 1 ng	22.29	2.83E+01	2.70E+08		
VI-WW3x 1 ng	21.87	2.83E+01	3.30E+08	4.58E+08	1.60E+08
VI-WW3x 1 ng	21.7	2.83E+01	3.59E+08		
VI-WW3x 10 ng	22.68	2.83E+01	2.24E+07		
VI-WW3x 10 ng	20.64	2.83E+01	5.94E+07		
VI-WW3x 10 ng	24.24	2.83E+01	1.06E+07		
VI-BW 0.5 ng	Undetermined	8.92E+01	0.00E+00		
VI-BW 0.5 ng	Undetermined	8.92E+01	0.00E+00		
VI-BW 0.5 ng	Undetermined	8.92E+01	0.00E+00	0.00E+00	0.00E+00
VI-BW 1 ng	Undetermined	8.92E+01	0.00E+00		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-BW 1 ng	Undetermined	8.92E+01	0.00E+00		
VI-BW 1 ng	Undetermined	8.92E+01	0.00E+00		
VI-BWx 0.5 ng	25.1	1.83E+01	1.19E+08		
VI-BWx 0.5 ng	24.82	1.83E+01	1.35E+08		
VI-BWx 0.5 ng	24.74	1.83E+01	1.41E+08		
VI-BWx 1 ng	22.83	1.83E+01	1.69E+08		
VI-BWx 1 ng	24.16	1.83E+01	9.15E+07	8.06E+07	6.67E+07
VI-BWx 1 ng	24.75	1.83E+01	6.99E+07		
VI-BWx 10 ng	Undetermined	1.83E+01	0.00E+00		
VI-BWx 10 ng	36.75	1.83E+01	2.80E+04		
VI-BWx 10 ng	Undetermined	1.83E+01	0.00E+00		
VII-BF2 0.5 ng	Undetermined	129.84	0.00E+00		
VII-BF2 0.5 ng	Undetermined	129.84	0.00E+00		
VII-BF2 0.5 ng	Undetermined	129.84	0.00E+00	0.00E+00	0.00E+00
VII-BF2 1 ng	Undetermined	129.84	0.00E+00		
VII-BF2 1 ng	Undetermined	129.84	0.00E+00		
VII-BF2 1 ng	Undetermined	129.84	0.00E+00		
VII-BF2x 0.5 ng	24.02	17.23	1.98E+08		
VII-BF2x 0.5 ng	24.33	17.23	1.71E+08		
VII-BF2x 0.5 ng	24.33	17.23	1.72E+08		
VII-BF2x 1 ng	23.32	17.23	1.36E+08		
VII-BF2x 1 ng	22.76	17.23	1.75E+08	1.65E+08	2.35E+07
VII-BF2x 1 ng	23.26	17.23	1.40E+08		
VII-BF2x 10 ng	32.53	17.23	2.06E+05		
VII-BF2x 10 ng	Undetermined	17.23	0.00E+00		
VII-BF2x 10 ng	30.93	17.23	4.27E+05		
VII-WW1 0.5 ng	35.83				
VII-WW1 0.5 ng	33.17	10.37	6.17E+05		
VII-WW1 0.5 ng	33.01	10.37	6.63E+05	5.13E+05	1.50E+05
VII-WW1 1 ng	32.04	10.37	5.16E+05		
VII-WW1 1 ng	32.14	10.37	4.92E+05		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VII-WW1 1 ng	33.41	10.37	2.77E+05		
VII-WW1x 0.5 ng	21.59	15.91	5.51E+08		
VII-WW1x 0.5 ng	20.82	15.91	7.81E+08		
VII-WW1x 0.5 ng	21.04	15.91	7.06E+08		
VII-WW1x 1 ng	20.65	15.91	4.22E+08		
VII-WW1x 1 ng	20.77	15.91	3.98E+08	5.54E+08	1.58E+08
VII-WW1x 1 ng	20.45	15.91	4.65E+08		
VII-WW1x 10 ng	18.99	15.91	8.99E+07		
VII-WW1x 10 ng	35.02	15.91	6.13E+04		
VII-WW1x 10 ng	Undetermined	15.91	0.00E+00		
VII-WW2 0.5 ng	35.19	9.69	2.30E+05		
VII-WW2 0.5 ng	34.86	9.69	2.67E+05		
VII-WW2 0.5 ng	35.59	9.69	1.92E+05	2.03E+05	4.50E+04
VII-WW2 1 ng	34.43	9.69	1.63E+05		
VII-WW2 1 ng	34.41	9.69	1.64E+05		
VII-WW2 1 ng	36.81				
VII-WW2x 0.5 ng	21.29	14.01	5.57E+08		
VII-WW2x 0.5 ng	21.2	14.01	5.81E+08		
VII-WW2x 0.5 ng	21.3	14.01	5.55E+08		
VII-WW2x 1 ng	19.67	14.01	5.81E+08		
VII-WW2x 1 ng	19.64	14.01	5.88E+08	5.73E+08	1.40E+07
VII-WW2x 1 ng	19.68	14.01	5.78E+08		
VII-WW2x 10 ng	18.92	14.01	8.16E+07		
VII-WW2x 10 ng	20.55	14.01	3.89E+07		
VII-WW2x 10 ng	25.61	14.01	3.90E+06		
VII-WW3 0.5 ng	Undetermined	2.57	0.00E+00		
VII-WW3 0.5 ng	Undetermined	2.57	0.00E+00		
VII-WW3 0.5 ng	Undetermined	2.57	0.00E+00	0.00E+00	0.00E+00
VII-WW3 1 ng	Undetermined	2.57	0.00E+00		
VII-WW3 1 ng	Undetermined	2.57	0.00E+00		
VII-WW3 1 ng	Undetermined	2.57	0.00E+00		
VII-WW3x 0.5 ng	21.61	26.21	9.01E+08		
				6.34E+08	3.81E+08

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VII-WW3x 0.5 ng	21.78	26.21	8.34E+08		
VII-WW3x 0.5 ng	21.48	26.21	9.53E+08		
VII-WW3x 1 ng	20.19	26.21	8.58E+08		
VII-WW3x 1 ng	20.4	26.21	7.80E+08		
VII-WW3x 1 ng	20.69	26.21	6.81E+08		
VII-WW3x 10 ng	27.76				
VII-WW3x 10 ng	22.92	26.21	2.48E+07		
VII-WW3x 10 ng	22.04	26.21	3.70E+07		

Table C.8.: Cells detected with *Arcobacter*-specific qPCR-Assay targeting the 23S rDNA gene in spiked and unspiked samples of sampling VIa-04/2010 VI-06/2010 VII-06/2010. Samples designated with "x" were spiked with 10^8 cells of *A. cryaerophilus*.

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-BW 0.5 ng	Undetermined	8.92E+01	0.00E+00		
VI-BW 0.5 ng	Undetermined	8.92E+01	0.00E+00		
VI-BW 0.5 ng	Undetermined	8.92E+01	0.00E+00	0.00E+00	0.00E+00
VI-BW 1 ng	Undetermined	8.92E+01	0.00E+00		
VI-BW 1 ng	Undetermined	8.92E+01	0.00E+00		
VI-BW 1 ng	Undetermined	8.92E+01	0.00E+00		
VI-BWx 0.5 ng	36.75	1.83E+01	4.47E+03		
VI-BWx 0.5 ng	36.5	1.83E+01	5.39E+03		
VI-BWx 0.5 ng	39.78	removed from analysis		4.17E+03	1.18E+03
VI-BWx 1 ng	36.57	1.83E+01	2.55E+03		
VI-BWx 1 ng	35.88	1.83E+01	4.28E+03		
VI-BWx 1 ng	39.99				
VI-BWx 10 ng	Undetermined			removed from	
VI-BWx 10 ng	Undetermined			analysis	
VI-BWx 10 ng	Undetermined				

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-S0 0.5 ng	Undetermined	2.09E+01	0.00E+00		
VI-S0 0.5 ng	Undetermined	2.09E+01	0.00E+00		
VI-S0 0.5 ng	Undetermined	2.09E+01	0.00E+00	0.00E+00	0.00E+00
VI-S0 1 ng	Undetermined	2.09E+01	0.00E+00		
VI-S0 1 ng	Undetermined	2.09E+01	0.00E+00		
VI-S0 1 ng	Undetermined	2.09E+01	0.00E+00		
VI-S0x 0.5 ng	38.66	2.31E+01			
VI-S0x 0.5 ng	36.79	2.31E+01			
VI-S0x 0.5 ng	Undetermined	2.31E+01			
VI-S0x 1 ng	29.31	2.31E+01	7.49E+05		
VI-S0x 1 ng	28.63	2.31E+01	1.25E+06	2.00E+08	2.30E+08
VI-S0x 1 ng	29.67	2.31E+01	5.71E+05		
VI-S0x 10 ng	17.65	2.31E+01	4.66E+08		
VI-S0x 10 ng	17.65	2.31E+01	4.66E+08		
VI-S0x 10 ng	18.4	2.31E+01	2.65E+08		
VI-S1 0.5 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1 0.5 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1 0.5 ng	Undetermined	3.17E+01	0.00E+00	0.00E+00	0.00E+00
VI-S1 1 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1 1 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1 1 ng	Undetermined	3.17E+01	0.00E+00		
VI-S1x 0.5 ng	30.24	2.43E+01	2.60E+05		
VI-S1x 0.5 ng	29.45	2.43E+01	4.69E+05		
VI-S1x 0.5 ng					
VI-S1x 1 ng	28.89	2.43E+01	1.07E+06		
VI-S1x 1 ng	27.54	2.43E+01	2.95E+06	5.76E+06	7.84E+06
VI-S1x 1 ng	28.54	2.43E+01	1.mat+06		
VI-S1x 10 ng	22.26	2.43E+01	1.55E+07		
VI-S1x 10 ng					
VI-S1x 10 ng	22.01	2.43E+01	1.87E+07		
VI-S2 0.5 ng	Undetermined	1.97E+01	0.00E+00		
				0.00E+00	0.00E+00

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-S2 0.5 ng	Undetermined	1.97E+01	0.00E+00		
VI-S2 0.5 ng	Undetermined	1.97E+01	0.00E+00		
VI-S2 1 ng	28.94	1.97E+01	0.00E+00		
VI-S2 1 ng	30.71	1.97E+01	0.00E+00		
VI-S2 1 ng	Undetermined	1.97E+01	0.00E+00		
VI-S2x 0.5 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 0.5 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 0.5 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 1 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 1 ng	Undetermined	2.62E+01	0.00E+00	0.00E+00	0.00E+00
VI-S2x 1 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 10 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 10 ng	Undetermined	2.62E+01	0.00E+00		
VI-S2x 10 ng	Undetermined	2.62E+01	0.00E+00		
VI-S3			not analyzed		
VI-S3x 0.5 ng	17.85	7.48E+00	1.61E+08		
VI-S3x 0.5 ng	17.85	7.48E+00	1.61E+08		
VI-S3x 0.5 ng	18.56	7.48E+00	9.53E+07	1.28E+08	2.67E+07
VI-S3x 1 ng	17.35	7.48E+00	1.16E+08		
VI-S3x 1 ng	17.3	7.48E+00	1.20E+08		
VI-S3x 1 ng	17.34	7.48E+00	1.17E+08		
VI-S3x 7.5 ng	16.44			removed from analysis	
VI-S3x 7.5 ng	14.89				
VI-S3x 7.5 ng	34.66				
VI-S4 0.5 ng	Undetermined	8.10E+01	0.00E+00		
VI-S4 0.5 ng	Undetermined	8.10E+01	0.00E+00		
VI-S4 0.5 ng	Undetermined	8.10E+01	0.00E+00	0.00E+00	0.00E+00
VI-S4 1 ng	Undetermined	8.10E+01	0.00E+00		
VI-S4 1 ng	Undetermined	8.10E+01	0.00E+00		
VI-S4 1 ng	Undetermined	8.10E+01	0.00E+00		
VI-S4x 0.5 ng	22.78	1.59E+01	9.27E+06		
VI-S4x 0.5 ng	22.75	1.59E+01	9.50E+06		
				1.23E+08	9.00E+07

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-S4x 0.5 ng	23.23	1.59E+01	6.66E+06		
VI-S4x 1 ng	17.75	1.59E+01	1.84E+08		
VI-S4x 1 ng	17.59	1.59E+01	2.08E+08		
VI-S4x 1 ng	18.1	1.59E+01	1.43E+08		
VI-S4x 10 ng	14.53	1.59E+01	1.95E+08		
VI-S4x 10 ng	14.39	1.59E+01	2.16E+08		
VI-S4x 10 ng	15.04	1.59E+01	1.34E+08		
VI-TW 0.4 ng	Undetermined	3.92E-01	0.00E+00		
VI-TW 0.4 ng	Undetermined	3.92E-01	0.00E+00	0.00E+00	0.00E+00
VI-TW 0.4 ng	Undetermined	3.92E-01	0.00E+00		
VI-TWx 0.5 ng	20.75	2.34E+01	3.90E+07		
VI-TWx 0.5 ng	21.49	2.34E+01	2.33E+07		
VI-TWx 0.5 ng	20.08	2.34E+01	6.21E+07		
VI-TWx 1 ng	17.61	2.34E+01	1.71E+08		
VI-TWx 1 ng	18.52	2.34E+01	9.10E+07	1.20E+08	7.77E+07
VI-TWx 1 ng	18.55	2.34E+01	8.94E+07		
VI-TWx 10 ng	14.03	2.34E+01	2.05E+08		
VI-TWx 10 ng	13.75	2.34E+01	2.49E+08		
VI-TWx 10 ng	14.49	2.34E+01	1.49E+08		
VI-S3 1 ng	Undetermined	3.27E+01	0.00E+00		
VI-S3 1 ng	Undetermined	3.27E+01	0.00E+00		
VI-S3 1 ng	Undetermined	3.27E+01	0.00E+00	0.00E+00	0.00E+00
VI-S3 0.5 ng	Undetermined	3.27E+01	0.00E+00		
VI-S3 0.5 ng	Undetermined	3.27E+01	0.00E+00		
VI-S3 0.5 ng	Undetermined	3.27E+01	0.00E+00		
VI-S3x			not analyzed		
VI-WW1 0.5 ng	Undetermined	5.45E+01	0.00E+00		
VI-WW1 0.5 ng	Undetermined	5.45E+01	0.00E+00		
VI-WW1 0.5 ng	Undetermined	5.45E+01	0.00E+00	0.00E+00	0.00E+00
VI-WW1 1 ng	Undetermined	5.45E+01	0.00E+00		
VI-WW1 1 ng	Undetermined	5.45E+01	0.00E+00		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-WW1 1 ng	Undetermined	5.45E+01	0.00E+00		
VI-WW1x 0.5 ng	19.31	1.72E+01	7.74E+07		
VI-WW1x 0.5 ng	19.09	1.72E+01	8.99E+07		
VI-WW1x 0.5 ng	19.ma	1.72E+01	5.73E+07		
VI-WW1x 1 ng	17.91	1.72E+01	1.02E+08		
VI-WW1x 1 ng	17.5	1.72E+01	1.36E+08	9.68E+07	2.72E+07
VI-WW1x 1 ng	17.87	1.72E+01	1.05E+08		
VI-WW1x 10 ng	14.21	1.72E+01	1.32E+08		
VI-WW1x 10 ng	15.23	1.72E+01	6.52E+07		
VI-WW1x 10 ng	14.51	1.72E+01	1.08E+08		
VI-WW2 0.5 ng	Undetermined	1.50E+02	0.00E+00		
VI-WW2 0.5 ng	Undetermined	1.50E+02	0.00E+00		
VI-WW2 0.5 ng	Undetermined	1.50E+02	0.00E+00	0.00E+00	0.00E+00
VI-WW2 1 ng	Undetermined	1.50E+02	0.00E+00		
VI-WW2 1 ng	Undetermined	1.50E+02	0.00E+00		
VI-WW2 1 ng	Undetermined	1.50E+02	0.00E+00		
VI-WW2x 0.5 ng	25.58	3.34E+01	1.96E+06		
VI-WW2x 0.5 ng	25.42	3.34E+01	2.19E+06		
VI-WW2x 0.5 ng	Undetermined	3.34E+01	0.00E+00		
VI-WW2x 1 ng	23.67	3.34E+01	3.68E+06		
VI-WW2x 1 ng	22.65	3.34E+01	7.46E+06	4.54E+07	6.54E+07
VI-WW2x 1 ng		3.34E+01	0.00E+00		
VI-WW2x 10 ng	15.25	3.34E+01	1.25E+08		
VI-WW2x 10 ng	15.42	3.34E+01	1.11E+08		
VI-WW2x 10 ng	14.92	3.34E+01	1.57E+08		
VI-WW3 0.5 ng	Undetermined	1.29E+02	0.00E+00		
VI-WW3 0.5 ng	Undetermined	1.29E+02	0.00E+00		
VI-WW3 0.5 ng	Undetermined	1.29E+02	0.00E+00	0.00E+00	0.00E+00
VI-WW3 1 ng	Undetermined	1.29E+02	0.00E+00		
VI-WW3 1 ng	Undetermined	1.29E+02	0.00E+00		
VI-WW3 1 ng	Undetermined	1.29E+02	0.00E+00		
VI-WW3x 0.5 ng	26.37	2.83E+01	9.63E+05		
				7.31E+07	7.68E+07

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-WW3x 0.5 ng	27.33	2.83E+01	4.93E+05		
VI-WW3x 0.5 ng	34.09	removed from analysis			
VI-WW3x 1 ng	ma.15	2.83E+01	3.57E+07		
VI-WW3x 1 ng	21.4	2.83E+01	1.50E+07		
VI-WW3x 1 ng	23.9	removed from analysis			
VI-WW3x 10 ng	14.51	2.83E+01	1.77E+08		
VI-WW3x 10 ng	14.89	2.83E+01	1.36E+08		
VI-WW3x 10 ng	14.79	2.83E+01	1.46E+08		
VIa-WW2 0.5 ng	Undetermined	7.15	0.00E+00		
VIa-WW2 0.5 ng	Undetermined	7.15	0.00E+00		
VIa-WW2 0.5 ng	Undetermined	7.15	0.00E+00	0.00E+00	0.00E+00
VIa-WW2 1 ng	Undetermined	7.15	0.00E+00		
VIa-WW2 1 ng	Undetermined	7.15	0.00E+00		
VIa-WW2 1 ng	Undetermined	7.15	0.00E+00		
VIa-WW2x 0.5 ng	22.47	8.98	1.97E+07		
VIa-WW2x 0.5 ng	21.4	8.98	4.29E+07		
VIa-WW2x 0.5 ng	22.33	8.98	2.18E+07	1.66E+07	1.51E+07
VIa-WW2x 1 ng	23.23	8.98	5.65E+06		
VIa-WW2x 1 ng	23.02	8.98	6.60E+06		
VIa-WW2x 1 ng	24.23	8.98	2.71E+06		
VIa-WW2x 9 ng	Undetermined			memoved from analysis	
VIa-WW2x 9 ng	21.03				
VIa-WW2x 9 ng	19.85				
VIa-WW3 0.5 ng	Undetermined	6.10	0.00E+00		
VIa-WW3 0.5 ng	Undetermined	6.10	0.00E+00		
VIa-WW3 0.5 ng	Undetermined	6.10	0.00E+00	0.00E+00	0.00E+00
VIa-WW3 1 ng	Undetermined	6.10	0.00E+00		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VIa-WW3 1 ng	Undetermined	6.10	0.00E+00		
VIa-WW3 1 ng	Undetermined	6.10	0.00E+00		
VIa-WW3x 0.5 ng	21.03	9.26	5.80E+07		
VIa-WW3x 0.5 ng	20.36	9.26	9.47E+07		
VIa-WW3x 0.5 ng	20.41	9.26	9.17E+07	1.35E+08	8.16E+07
VIa-WW3x 1 ng	17.93	9.26	2.82E+08		
VIa-WW3x 1 ng	19.21	9.26	1.10E+08		
VIa-WW3x 1 ng	18.58	9.26	1.75E+08		
VIa-WW3x 9 ng	14.87				
VIa-WW3x 9 ng	15.33			removed from analysis	
VIa-WW3x 9 ng	15.68				
VII-WW1 0.5 ng	Undetermined	1.04E+01	0.00E+00		
VII-WW1 0.5 ng	Undetermined	1.04E+01	0.00E+00		
VII-WW1 0.5 ng	Undetermined	1.04E+01	0.00E+00	0.00E+00	0.00E+00
VII-WW1 1 ng	Undetermined	1.04E+01	0.00E+00		
VII-WW1 1 ng	Undetermined	1.04E+01	0.00E+00		
VII-WW1 1 ng	Undetermined	1.04E+01	0.00E+00		
VII-WW1x 0.5 ng	23.05	1.72E+01	9.18E+07		
VII-WW1x 0.5 ng	23.56	1.72E+01	6.23E+07		
VII-WW1x 0.5 ng	22.81	1.72E+01	1.11E+08	1.19E+08	5.22E+07
VII-WW1x 1 ng	21.11	1.72E+01	2.01E+08		
VII-WW1x 1 ng	21.72	1.72E+01	1.27E+08		
VII-WW1x 1 ng	23.2				
VII-WW1x 10 ng	26.37			removed from	
VII-WW1x 10 ng	17.34			analysis	
VII-WW1x 10 ng	17.02				
VII-WW2 0.5 ng	Undetermined	9.69E+00	0.00E+00		
VII-WW2 0.5 ng	Undetermined	9.69E+00	0.00E+00		
VII-WW2 0.5 ng	Undetermined	9.69E+00	0.00E+00	0.00E+00	0.00E+00
VII-WW2 1 ng	Undetermined	9.69E+00	0.00E+00		
VII-WW2 1 ng	Undetermined	9.69E+00	0.00E+00		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VII-WW2 1 ng	Undetermined	9.69E+00	0.00E+00		
VII-WW2x 0.5 ng	23.76	3.34E+01	1.04E+08		
VII-WW2x 0.5 ng	23.99	3.34E+01	8.72E+07		
VII-WW2x 0.5 ng	25.45	3.34E+01	2.86E+07		
VII-WW2x 1 ng	21.75	3.34E+01	2.42E+08		
VII-WW2x 1 ng	20.34	3.34E+01	7.08E+08	5.64E+08	4.79E+08
VII-WW2x 1 ng	20.57	3.34E+01	5.93E+08		
VII-WW2x 10 ng	16.63	3.34E+01	1.19E+09		
VII-WW2x 10 ng	16.54	3.34E+01	1.28E+09		
VII-WW2x 10 ng	17.08	3.34E+01	8.44E+08		
VII-WW3 0.5 ng	Undetermined	2.57E+00	0.00E+00		
VII-WW3 0.5 ng	Undetermined	2.57E+00	0.00E+00		
VII-WW3 0.5 ng	Undetermined	2.57E+00	0.00E+00	0.00E+00	0.00E+00
VII-WW3 1 ng	Undetermined	2.57E+00	0.00E+00		
VII-WW3 1 ng	Undetermined	2.57E+00	0.00E+00		
VII-WW3 1 ng	Undetermined	2.57E+00	0.00E+00		
VII-WW3x 0.5 ng	24.86	2.83E+01	3.81E+07		
VII-WW3x 0.5 ng	23.58	2.83E+01	1.01E+08		
VII-WW3x 0.5 ng	23.27	2.83E+01	1.28E+08	1.46E+08	7.28E+07
VII-WW3x 1 ng	21.73	2.83E+01	2.06E+08		
VII-WW3x 1 ng	22.01	2.83E+01	1.67E+08		
VII-WW3x 1 ng	21.55	2.83E+01	2.38E+08		
VII-WW3x 10 ng	18.51				
VII-WW3x 10 ng	17.25			removed from analysis	
VII-WW3x 10 ng	21.41				
VII-BF1 0.5 ng	Undetermined	3.60E+02	0.00E+00		
VII-BF1 0.5 ng	Undetermined	3.60E+02	0.00E+00		
VII-BF1 0.5 ng	Undetermined	3.60E+02	0.00E+00		
VII-BF1 1 ng	Undetermined	3.60E+02	0.00E+00	0.00E+00	0.00E+00
VII-BF1 1 ng	Undetermined	3.60E+02	0.00E+00		
VII-BF1 1 ng	Undetermined	3.60E+02	0.00E+00		
VII-BF1 0.5 ng	Undetermined	5.01E+01	0.00E+00		

Sample	C_t	gDNA conc. $[ng * \mu l^{-1}]$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VII-BF1x 0.5 ng	Undetermined	5.01E+01	0.00E+00		
VII-BF1x 0.5 ng	Undetermined	5.01E+01	0.00E+00	0.00E+00	0.00E+00
VII-BF1x 0.5 ng	Undetermined	5.01E+01	0.00E+00		
VII-BF1x 1 ng	39.42			removed from	
VII-BF1x 1 ng	Undetermined			analysis	
VII-BF1x 10 ng	19.6				
VII-BF1x 10 ng	20.18	5.01E+01	1.19E+08	4.28E+07	6.94E+07
VII-BF1x 10 ng	20.7	5.01E+01	8.04E+07		
VII-BF2 0.5 ng	Undetermined	15.50	0.00E+00		
VII-BF2 0.5 ng	Undetermined	15.50	0.00E+00		
VII-BF2 0.5 ng	Undetermined	15.50	0.00E+00	0.00E+00	0.00E+00
VII-BF2 1 ng	Undetermined	15.50	0.00E+00		
VII-BF2 1 ng	Undetermined	15.50	0.00E+00		
VII-BF2 1 ng	Undetermined	15.50	0.00E+00		
VII-BF2x 0.5 ng	24.56				
VII-BF2x 0.5 ng	26.41			removed from analysis	
VII-BF2x 0.5 ng	28.89				
VII-BF2x 1 ng	23.22	15.50	1.38E+06		
VII-BF2x 1 ng	23.54	15.50	1.08E+06	1.43E+06	3.81E+05
VII-BF2x 1 ng	22.84	15.50	1.84E+06		
VII-BF2x 10 ng	Undetermined				
VII-BF2x 10 ng	Undetermined			removed from analysis	
VII-BF2x 10 ng	20.38				

Table C.9.: Cells detected with *Arcobacter*-specific qPCR-Assay targeting the 23S rDNA gene in enrichment cultures of samples from samplings VI-06/2010 and VII-06/2010.

Sample	C_t	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-En-WW1	20.05	7.83E+07		
VI-En-WW1	20.1	7.54E+07	6.27E+07	2.45E+07
VI-En-WW1	21.13	3.45E+07		
VI-En-WW2	30.33	3.24E+04		
VI-En-WW2	31.03	1.91E+04	2.20E+04	9.33E+03
VI-En-WW2	31.4	1.44E+04		
VI-En-WW3	21.86	1.99E+07	removed from analysis	
VI-En-WW3	Undetermined	0.00E+00		
VI-En-WW3	30.87	2.15E+04		
VI-En-BW	Undetermined	0.00E+00		
VI-En-BW	Undetermined	0.00E+00	0.00E+00	0.00E+00
VI-En-BW	Undetermined	0.00E+00		
VI-En-TW	Undetermined	0.00E+00		
VI-En-TW	Undetermined	0.00E+00	0.00E+00	0.00E+00
VI-En-TW	Undetermined	0.00E+00		
VI-En-S0	Undetermined	0.00E+00		
VI-En-S0	Undetermined	0.00E+00	0.00E+00	0.00E+00
VI-En-S0	Undetermined	0.00E+00		
VI-En-S1	Undetermined	0.00E+00		
VI-En-S1	Undetermined	0.00E+00	0.00E+00	0.00E+00
VI-En-S1	Undetermined	0.00E+00		
VI-En-S2	Undetermined	0.00E+00		
VI-En-S2	Undetermined	0.00E+00	0.00E+00	0.00E+00
VI-En-S2	Undetermined	0.00E+00		
VI-En-S3	Undetermined	0.00E+00		
			0.00E+00	0.00E+00

Sample	C_t	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
VI-En-S3	Undetermined	0.00E+00		
VI-En-S3	Undetermined	0.00E+00		
VI-En-S4	28.32	1.49E+05	removed from analysis	
VI-En-S4	Undetermined	0.00E+00		
VI-En-S4	24.18	3.41E+06		
VII-En-WW1	22.38	1.34E+07	1.41E+07	9.92E+05
VII-En-WW1	22.25	1.48E+07		
VII-En-WW1	25.64		removed from analysis	
VII-En-WW2	23.14	7.52E+06		
VII-En-WW2	23.78	4.63E+06	7.55E+06	2.94E+06
VII-En-WW2	22.7	1.05E+07		
VII-En-WW3	30.13	3.78E+04		
VII-En-WW3	25.61	1.16E+06	0.00E+00	0.00E+00
VII-En-WW3	33.29	3.44E+03		
VII-En-BF2	23.57	5.44E+06		
VII-En-BF2	23.29	6.75E+06	5.67E+06	9.84E+05
VII-En-BF2	23.73	4.82E+06		

C.5. Detection of *Pectobacterium* spp. by qPCR

Table C.10.: Cells detected with a *Pectobacterium*-specific qPCR-assay targeting the *mdh*-gene in samples of samplings IV-07/2009 and V-10/2009.

Sample	C_t	gDNA conc. $ng * \mu l^{-1}$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
IV-S0 1 ng	25.76	3.62E+01	4.30E+04		
IV-S0 1 ng	25.76	3.62E+01	4.31E+04		
IV-S0 1 ng	25.70	3.62E+01	4.53E+04		
IV-S0 0.5 ng	25.99	3.62E+01	7.26E+04	5.57E+04	1.38E+04
IV-S0 0.5 ng	26.24	3.62E+01	5.96E+04		
IV-S0 0.5 ng	26.03	3.62E+01	7.04E+04		
IV-S1 1 ng	25.89	5.46E+01	5.91E+04		
IV-S1 1 ng	26.12	5.46E+01	4.94E+04		
IV-S1 1 ng	26.16	5.46E+01	4.78E+04	6.47E+04	1.85E+04
IV-S1 0.5 ng	26.24	5.46E+01	8.98E+04		
IV-S1 0.5 ng	26.30	5.46E+01	8.61E+04		
IV-S1 0.5 ng	26.86	5.46E+01	5.61E+04		
IV-S2	26.97	3.31E+00	9.86E+03		
IV-S2 1 ng	27.14	3.31E+00	8.63E+03		
IV-S2 1 ng	27.16	3.31E+00	8.54E+03	8.13E+03	1.08E+03
IV-S2 0.5 ng	28.29	3.31E+00	7.33E+03		
IV-S2 0.5 ng	28.34	3.31E+00	7.03E+03		
IV-S2 0.5 ng	28.28	3.31E+00	7.37E+03		
IV-S3 1 ng	23.92	7.54E+00	3.69E+04		
IV-S3 1 ng	24.54	7.54E+00	2.30E+04		
IV-S3 1 ng	23.43	7.54E+00	5.39E+04	5.73E+04	2.59E+04
IV-S3 0.5 ng	23.57	7.54E+00	9.71E+04		
IV-S3 0.5 ng	24.10	7.54E+00	6.45E+04		
IV-S3 0.5 ng	24.03	7.54E+00	6.82E+04		
IV-S4 1 ng	29.94	5.22E+00	1.68E+03		
IV-S4 1 ng	30.31	5.22E+00	1.27E+03		
				1.35E+03	2.53E+02

Sample	C_t	gDNA conc. $ng * \mu l^{-1}$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
IV-S0 1 ng	25.76	3.62E+01	4.30E+04		
IV-S4 1 ng	30.14	5.22E+00	1.45E+03		
IV-S4 0.5 ng	31.34	5.22E+00	1.18E+03		
IV-S4 0.5 ng	31.59	5.22E+00	9.82E+02		
IV-S4 0.5 ng	30.99	5.22E+00	1.53E+03		
IV-TW 1 ng	33.23	5.73E+00	1.57E+02		
IV-TW 1 ng	32.56	5.73E+00	2.59E+02	2.04E+02	5.13E+01
IV-TW 1 ng	32.94	5.73E+00	1.96E+02		
IV-WW1 1 ng	27.50	8.28E+01	2.59E+04		
IV-WW1 1 ng	27.61	8.28E+01	2.38E+04		
IV-WW1 1 ng	27.86	8.28E+01	1.96E+04	2.76E+04	5.49E+03
IV-WW1 0.5 ng	28.18	8.28E+01	3.08E+04		
IV-WW1 0.5 ng	28.06	8.28E+01	3.38E+04		
IV-WW1 0.5 ng	28.12	8.28E+01	3.21E+04		
IV-WW2 1 ng	25.55	6.05E+01	8.49E+04		
IV-WW2 1 ng	25.63	6.05E+01	7.97E+04		
IV-WW2 1 ng	25.49	6.05E+01	8.85E+04	1.11E+05	3.22E+04
IV-WW2 0.5 ng	26.04	6.05E+01	1.16E+05		
IV-WW2 0.5 ng	25.77	6.05E+01	1.43E+05		
IV-WW2 0.5 ng	25.66	6.05E+01	1.56E+05		
IV-WW3 1 ng	24.09	8.10E+02	6.89E+06		
IV-WW3 1 ng	24.87	8.10E+02	3.84E+06		
IV-WW3 1 ng	24.47	8.10E+02	5.19E+06	5.23E+06	9.76E+05
IV-WW3 0.5 ng	25.37	8.10E+02	5.30E+06		
IV-WW3 0.5 ng	25.41	8.10E+02	5.14E+06		
IV-WW3 0.5 ng	25.45	8.10E+02	5.00E+06		
IV-BW 1 ng	21.61	2.26E+01	6.56E+05		
IV-BW 1 ng	21.47	2.26E+01			
IV-BW 1 ng	21.26	2.26E+01		6.12E+05	1.68E+05
IV-BW 0.5 ng	23.04	2.26E+01			

Sample	C_t	gDNA conc. $ng * \mu l^{-1}$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
IV-S0 1 ng	25.76	3.62E+01	4.30E+04		
IV-BW 0.5 ng	23.00	2.26E+01			
IV-BW 0.5 ng	22.78	2.26E+01			
V-S0 1 ng	Undetermined	9.02E+00	0.00E+00		
V-S0 1 ng	Undetermined	9.02E+00	0.00E+00	0.00E+00	0.00E+00
V-S0 1 ng	Undetermined	9.02E+00	0.00E+00		
V-S1 1 ng	32.38	1.37E+00	7.07E+01		
V-S1 1 ng	32.24	1.37E+00	7.85E+01		
V-S1 1 ng	32.41	1.37E+00	6.96E+01	8.42E+01	1.76E+01
V-S1 0.5 ng	33.03	1.37E+00	8.67E+01		
V-S1 0.5 ng	32.63	1.37E+00	1.17E+02		
V-S1 0.5 ng	33.12	1.37E+00	8.19E+01		
V-S2 0.5 ng	Undetermined	5.88E-01	0.00E+00		
V-S2 0.5 ng	Undetermined	5.88E-01	0.00E+00	0.00E+00	0.00E+00
V-S2 0.5 ng	Undetermined	5.88E-01	0.00E+00		
V-S3 0.1 ng	28.24	1.28E+00	1.72E+03		
V-S3 0.1 ng	28.12	1.28E+00	1.89E+03	1.69E+03	2.15E+02
V-S3 0.1 ng	28.45	1.28E+00	1.47E+03		
V-S4 1 ng	Undetermined	4.15E+00	0.00E+00		
V-S4 1 ng	31.84	4.15E+00	3.56E+01	1.19E+01	2.06E+01
V-S4 1 ng	Undetermined	4.15E+00	0.00E+00		
V-TW 1 ng	Undetermined	2.63E-01	0.00E+00		
V-TW 1 ng	Undetermined	2.63E-01	0.00E+00		
V-TW 1 ng	Undetermined	2.63E-01	0.00E+00	0.00E+00	0.00E+00
V-TW 0.5 ng	Undetermined	2.63E-01	0.00E+00		
V-TW 0.5 ng	Undetermined	2.63E-01	0.00E+00		
V-TW 0.5 ng	Undetermined	2.63E-01	0.00E+00		
V-WW1 1 ng	35.55	1.35E+02	6.29E+01		
V-WW1 1 ng	37.07	1.35E+02	1.91E+01	4.18E+01	2.19E+01

Sample	C_t	gDNA conc. $ng * \mu l^{-1}$	Detected cell numbers per ml	Average cell numbers per ml	Stdev of cell numbers per ml
IV-S0 1 ng	25.76	3.62E+01	4.30E+04		
V-WW1 1 ng	36.02	1.35E+02	4.36E+01		
V-WW2 1 ng	34.39	1.35E+02	1.56E+02		
V-WW2 1 ng	33.50	1.35E+02	3.15E+02	2.12E+02	8.94E+01
V-WW2 1 ng	34.32	1.35E+02	1.65E+02		
V-WW3 1 ng	34.14	1.35E+02	1.91E+02		
V-WW3 1 ng	34.35	1.35E+02	1.61E+02	2.13E+02	6.49E+01
V-WW3 1 ng	33.62	1.35E+02	2.86E+02		
V-BW 1 ng	32.30	3.88E+01	2.31E+02		
V-BW 1 ng	32.08	3.88E+01	2.74E+02	2.37E+02	3.35E+01
V-BW 1 ng	32.43	3.88E+01	2.08E+02		

C.6. Establishment of the diversity by culture-dependent MALDI-TOF MS of a spinach-processing line

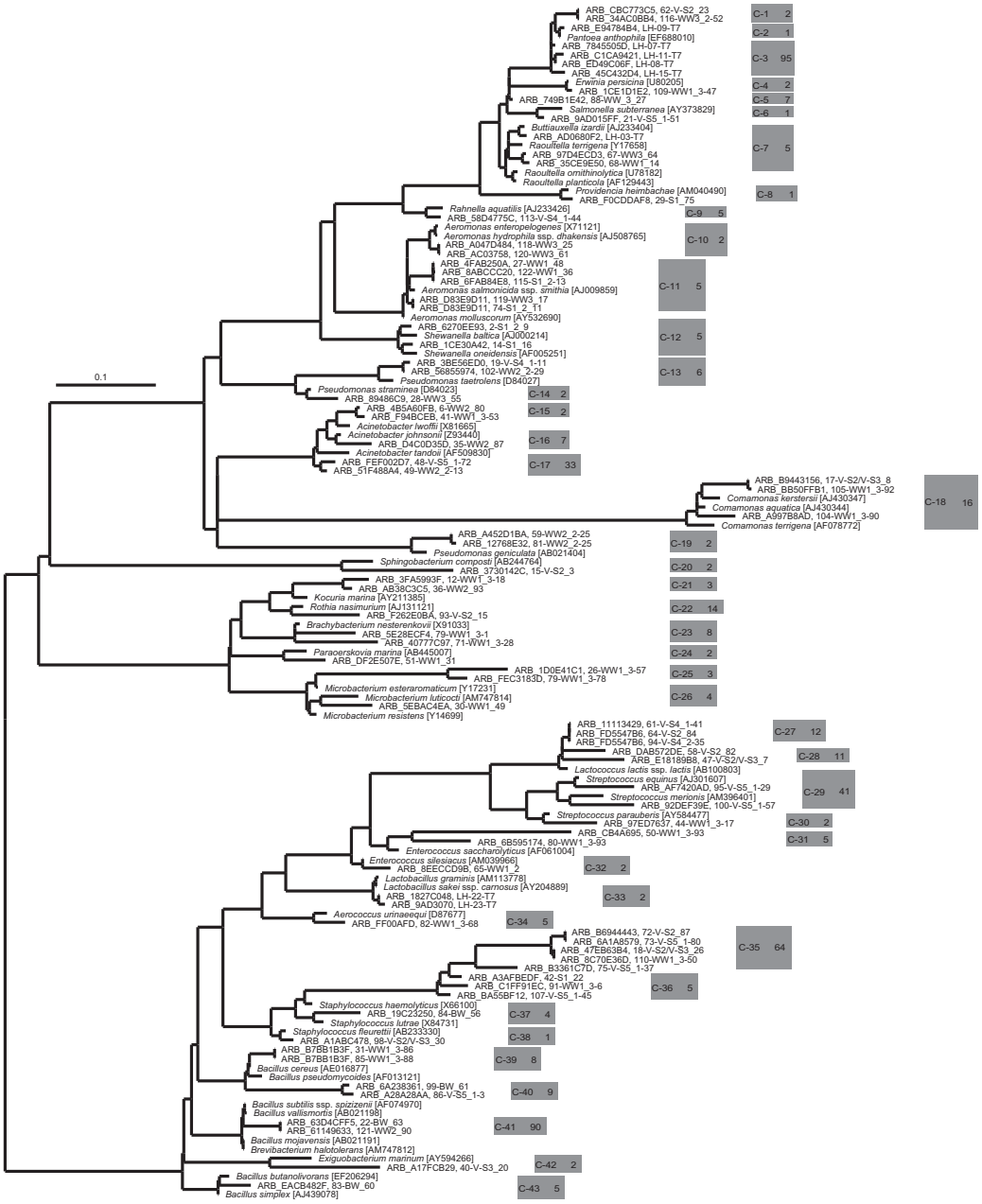

Figure C.5.: Phylogenetic affiliation of the strains identified by MALDI-TOF MS analysis based on their 16S rRNA gene sequences. The tree was constructed with the latest update of the all-species living tree project (LTPs102 release; Yarza et al. (2008)) and the Maximum Parsimony algorithm Kolaczkowski & Thornton (2004). In shadow is indicated each MALDI-TOF/MS phenotypic cluster with its cluster number and the number of isolates within each of them.

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